PHYSIOLOGICAL REVIEWS

VOLUME 19

BALTIMORE, MD.
1939

CONTENTS

No. 1. January, 1939

No. 2. April, 1939

No. 3. July, 1939

No. 4. October, 1939

PHYSIOLOGICAL REVIEWS

VOL. 19 JANUARY, 1939 No. 1

THE REACTION OF MUSCLE TO DENERVATION

SARAH S. TOWER

Department of Anatomy, Johns Hopkins University

The atrophy and degeneration of denervated skeletal muscle have generally been held to constitute the outstanding demonstration, by elimination, of trophic control of a non-nervous tissue by the nervous system. This review is primarily an inquiry into the nature of the changes taking place in denervated muscle, with the object of uncovering the mechanism of their production. The results of this inquiry will then be reinterpreted in an effort to establish the scope and character of the neurotrophic relationship previously operative. A priori, the trophic influence could be exercised by physical integrity of innervation, either by substantial transfer from nerve to non-nervous tissue of neurohumors or other materials, or by more subtle interaction. Or it could derive from activity in the non-nervous tissue of nervous initiation. Or, since the agency of trophic influence need not necessarily be single, both could coöperate.

The problem of mechanism in the production of atrophy and degeneration in muscle may be resolved into two major parts. The simpler of these concerns the question: which of the several components of peripheral innervation, motor, sensory, sympathetic, possibly parasympathetic (posterior root efferent or other) determine in their elimination the onset of the regressive changes. This is a separate problem for the extrafusal muscle fiber and for the two parts, polar and equatorial, of the intrafusal fiber. The more difficult problem concerns the nature of the changes resulting from interruption of any one or of all of the components of peripheral innervation. Most of the observations on the reaction of muscle to denervation relate to total denervation, and consider the extrafusal muscle only. These will be described first. The problem of the share of the components of peripheral innervation in this reaction will then be taken up, followed by the special observations and problems relating to the spindle. The facts thus marshalled will then

be analysed for the mechanism of the reaction of muscle to denervation. Finally, the nature of the normal neurotrophic relationship thus destroyed in denervation will be examined.

REACTION OF MUSCLE TO TOTAL DENERVATION. PHYSIOLOGICAL OBSERVATIONS. The outstanding functional result of denervating skeletal muscle is unquestionably the development of spontaneous, continuous, rhythmical contraction. This "fibrillation" is, however, delayed in onset for several days, while changes in the excitability of the muscle to direct and indirect stimulation, and in the form of contraction develop sooner. In general, physiological studies have concentrated on the fully developed reaction to denervation of the second to fourth week after nerve section, neglecting to examine the onset of the reaction in the very first days, while only scattered observations on man are available to complete the picture in its later stages.

Excitability: Changes in excitability were among the earliest recognized aspects of the reaction of muscle to denervation, involving a diminution of excitability to faradic current, and an increased excitability to galvanic current or to mechanical stimulation. In spite of this, there is still no thoroughgoing study of excitability in terms of the strength-duration function which covers the onset and long course of changes following maintained denervation of skeletal muscle. Richter (1936) has examined the function in a limited way during the first 48 hours after nerve section in rats. He found that after 6 hours the chronaxie of the muscle was definitely shortened, remained so for a few hours, became normal again for 24 to 36 hours and then began to rise. Holobut and Jalowy (1936), in a correlated morphological and physiological study beginning 15 hours after sciatic section in the rat, reported that up to 24 to 48 hours, when the motor end-plates in the gastrocnemius muscle were visably altered, both rheobase and chronaxie of the muscle were still normal. This would coincide with Richter's second normal interval. Thereafter as the nerve became inexcitable 3 to 5 days after section, the chronaxie of the denervated muscle began to increase. Neither of these studies, however, gives the full strength-duration curve; only figures for rheobase and chronaxie.

For the later development of changes in the excitability of denervated muscle the best data available are from man. Adrian (1917) published strength-duration curves taken from the 3rd to the 60th day after onset of a facial paralysis of unstated etiology. For the first 8 days the curves remained absolutely unchanged. From the 12th through the 15th days, and again after the 33rd day when signs of re-

generation appeared, the strength-duration curves showed a discontinuity, indicative of two mechanisms of excitation, one rapid and one slow. The rapid curve, which was the only curve up to the 12th day, was obtained with the cathode on the nerve as well as on the muscle, but its presence in the muscle as late as 14 days after the onset of paralysis hardly permits of its being ascribed to nerve alone. From the 16th day through the 28th day only the slow curve was obtained, and from its first appearance on the 12th day to the 28th day, this chronaxie, considered as of the muscle, increased from 0.008 to 0.032 second, showing that the process of atrophy may be attended by progressive lengthening of chronaxie. In an earlier study Adrian (1916) had already presented more evidence of similar import.

From Adrian's studies it appears that the short chronaxie of normal muscle in situ, which is also the chronaxie of the motor nerve, disappears completely after destruction of the nerve, but without preliminary modification. As this goes, a long chronaxie comes into effect which is in all cases many times (10 to 100) the normal. The value of this chronaxie is determined by the degree of wasting of the muscle such that in the early stages after denervation, while wasting is rapid, chronaxie lengthens progressively, while in later stages, when wasting is much slowed up and perhaps even at a standstill, chronaxie is correspondingly stable. The very late state of fibrosis and virtual inexcitability has not been examined for chronaxie, if indeed it could be. Adrian did not identify his strength-duration curves by the usual symbols, but the slow curve is obviously the α curve, and the fast, probably β. Although Adrian is alone in describing discontinuity of strength-duration function after denervation, he is also apparently alone in making the repeated examination of the full function which will demonstrate it.

Contractility: Denny-Brown (1929) fixed the onset of the degeneration of skeletal muscle as a contractile structure as early as two minutes after nerve section. At this time he found that the duration of contraction of cat's muscle in situ on nerve stimulation was appreciably lengthened, while parallel with the lengthening duration, the tension of maximal twitch or maximal tetanus fell off. After a rapid change in the first 2 to 10 minutes, further deterioration was slow. These results were obtained equally with peripheral nerve or ventral root section, de-afferentation having no influence. Since it is generally agreed that the nerve fibers themselves continue to function for considerably more than 24 hours, the loss of tension in this interval cannot represent dropping out of nerve fibers with corresponding reduction in the number

of muscle fibers contracting,). must represent a change in the contractility of the muscle fibe themselves. So far, these potentially extremely significant results re unconfirmed. Contrarily, Cook and Gerard (1931) detected no ing off in the maximal response of dog's muscle to nerve stimulatic rom the first half-hour to 30 to 40 hours after nerve section.

Toward the end of th rst week or beginning of the second week after nerve section, dir electrical stimulation of muscle brings out clearly the prolongatic f contraction which is characteristic of the response of denervate nuscle to forms of stimulation as diverse as mechanical, electrical pharmacodynamical, and which was, perhaps, prefigured in Denny own's results. Indirect excitability has failed some days previous Strong induced shocks, though not weak ones, show the prolonga but stimuli of longer duration are much more effective. In the rse of the second week two definite components of contraction beco unmistakable, a twitch as brisk as the response of normal muscle, owed by a sustained contraction, or contracture. Roberts (1916) orded the response of denervated rabbit's muscle to electrical s llation and showed that the two components are separable on basis either of threshold or of fatiguability, the brisk contraction g both more easily excited and fatigued. Red and white musc haved similarly in the form, in the time of onset, and in the rat development of the changes in contraction following denervati

The co ctile response to acetylcholine stimulation has been more thoroug xamined, particularly recently with the technique of close intra-a l injection (Brown, 1937; Rosenblueth and Luco, 1937). The fi nase of quick contraction resembles the response of normal musc acetylcholine in all except the very much smaller dosage requ o elicit it, and a longer duration. The second phase of slow con on has long been familiar as acetylcholine contracture. Again, th k contraction has the lower threshold, for small doses of acetylcl may excite only quick contraction, larger doses being required t oduce contracture.

e electrical activity of denervated muscle resembles that of normal le in the phase of quick contraction. Acetylcholine produces a f outburst as in a brief tetanus, and electrical stimulation, either a gle burst or a tetanus depending on whether single or repetitive imulation is applied. Rosenblueth and Luco likewise found that aroughout the prolonged contraction remainders of electrical stimula-

tion, spike potentials continue with increased amplitude and frequency. Acetylcholine contracture, on the contrary, follows on and curtails the quick contraction both mechanically and electrically, and may produce an electrical "silence" lasting ten minute or more, introducing a phenomenon of refractoriness which has so far not been observed with the other forms of stimulation described. However, the oscillographic recording systems used by Brown and by Rosenblueth and Luco were incapable of registering slow potential changes. Using a string galvanometer, Schäffer and Licht (1926) found that both acetylcholine contracture and the related pseudo-motor contracture are accompanied by a slow monophasic negative potential in muscle.

In addition to refractoriness, acetylcholine stimulation introduces a second special phenomenon in sensitization, whereby amounts of this substance which are entirely inadequate to excite normal muscle, suffice to excite both quick and slow contraction in denervated muscle. Other stimulating substances also show this (Dale and Gasser, 1926). This acetylcholine sensitization underlies both the artificially excited pseudomotor contractures of motorially denervated muscle (Dale and Gaddum, 1930), which depend upon intact vasodilator fibers, and the naturally occurring fright contraction recently observed by Bender (1938) in the denervated facial muscle of monkeys.

The time-course of development of the changes in contraction has never been carefully examined with direct electrical stimulation of muscle. More data have recently become available from acetylcholine stimulation, giving an onset much earlier than had previously been thought, preceding, indeed, or attending abolition of indirect excitability of the muscle. Obviously the quick contraction is carried over from the normal state, weakening rapidly, although just when it begins to weaken is not too clear. Brown (1937) reported that in the one cat examined on the 4th day after nerve section, in which the threshold for acetylcholine was not lower than half the normal value, the slow contraction was only just visible, while 6 days after denervation both sensitization and slow contraction appeared to be fully developed. Knowlton and Hines (1937) found for the rat that both acetylcholine sensitization and the slow contraction, or contracture, appeared between the 2nd and 3rd days after nerve section, reached after 7 days a maximum which was maintained for about 3 weeks, and then declined. Heidenhain's (1883) description of the time course of development of the pseudo-motor contracture in the dog's tongue accords almost exactly with these results of Knowlton and Hines: onset on the 4th day, maxi-

mum by the 8th or 9th, constancy to the end of the 3rd week, and then decline accompanying nerve regeneration. Unfortunately, the time-course of development of changes in contraction and in chronaxie have never been examined together, nor even separately but adequately in the same species. Nevertheless the correlation is unmistakable between weakening and disappearance of quick contraction and rapid chronaxie, and the development of slow contraction and of slow chronaxie.

After the second month the response of denervated muscle to direct electrical stimulation has been examined only in the intact animal or in man, with general agreement for both that it becomes progressively more sluggish. From this it may be inferred that the quick contraction weakens, and perhaps disappears completely. But the slow component must also finally weaken, because the power of contraction diminishes steadily as the muscle wastes, even to nothing. How long muscle can survive denervation is still an open question, but one which should be answered in terms of survival of contractility. Here the experimental animal has contributed little because observation has rarely been continued more than a few months, and contractility survives easily for this time. Of quite another order of magnitude are some durations of survival in man. Sherren (1908) has put on record a case in which the muscles reacted to constant current although the musculospiral nerve had been divided for 23 years. And Duel (1932) has reported two cases of facial paralysis of surgical origin of 13 and 18 years' duration, with verification at operation of the completeness of the nerve section in the latter, in which the facial muscle responded to galvanic current but not to faradic. After a repair operation, both cases made a demonstrable recovery of function, the longer standing case recovering the faradic response.

"Reaction of degeneration" in muscle: Since the time of Erb (1868, 1869) certain aspects of the changed response of muscle to electrical stimulation through the skin have been recognized and designated as the "reaction of degeneration," and considered pathognomonic of partial or complete denervation of the muscle. With stimulation of the nerve these are: increased excitability to faradic current 2 to 3 days after nerve injury, followed by diminished excitability to faradic and galvanic currents to the point of extinction after 7 to 12 days. With stimulation of the muscle at its motor point they are: diminished excitability to faradic current to the point of practical extinction in the second week; diminished excitability to galvanic current in the first week, followed by increased excitability and a change in the form of the response from a

brief twitch on make and break of the current, to a sluggish, sustained contraction; and by reversal such that the cathodal closing stimulus, normally the most effective, becomes less effective than the anodal closing stimulus. This state continues for some months, but is then followed by progressive diminution of excitability even to constant current unless regeneration sets in. Erb also described the increased mechanical irritability of denervated muscle. In man, the reaction of degeneration is usually clear-cut ten days after a peripheral nerve lesion, and similarly in the cat and dog. But in the rat (Hines and Knowlton, 1933), in which all aspects of change following denervation progress rapidly, the reaction of degeneration is typical as early as the third day.

"Reaction of degeneration" is primarily a clinical concept, with limitations imposed by clinical method which from a physiological point of view amount to error. Thus faradic excitability of the muscle is abolished in the second week only if the stimulus be applied and observation be made through the intact skin. If the muscle is stimulated and observed directly, a response to faradic stimulation may be obtained for months. The formula KCC>ACC, and its reversal by denervation, are also subject to special conditions. Roberts (1916) showed for man that the greater effectiveness of KCC over ACC holds only at the motor point of muscle,—that is, where the nerve is most accessible. Muscle tissue itself, normal or denervated, responds variably, but on the whole equally to KCC and ACC.

Fibrillation: Observed originally by Schiff in 1851, and carefully studied by him, noted again by Rogowicz (1885), and again by Ricker (1901) as "tremor" in denervated muscle, rediscovered and carefully studied again by Langley and Kato (1915a) and made by Langley the basis of his understanding of muscular atrophy, and repeatedly observed since, the phenomenon of spontaneous, continuous, rhythmical contraction in denervated muscle still has made remarkably little impression.

Except in the tongue, or as magnified by the length of the rabbit's or cat's whiskers (which are moved by striated muscle innervated by the VII nerve), the fibrillation of denervated muscle is visible in the experimental animal only on direct examination of the exposed muscle, preferably with strong, oblique illumination. It is not appreciably through the skin, nor even through a heavy fascia such as the antebrachial fascia. Elsewhere than in the tongue, denervation-fibrillation has generally not been recognized as such in human muscle. But recently, first

Grund (1937) discovered that the fibrillation in two cases of amyotrophic lateral sclerosis was not abolished by spinal anesthesia; then Russel, Odum and McEachern (1938) found that novocain block of the common peroneal nerve failed to influence the fibrillation in three cases of progressive muscular atrophy, forcing the conclusion in both these studies that the fibrillation was of peripheral origin. This raises the question concerning the fairly coarse fibrillation seen through the skin in many conditions in man: to what extent, or in what types of case is such fibrillation properly ascribed to irritative degeneration of anterior horn cells, with all the fibers innervated from one motor horn cell contracting synchronously; to what extent does it represent the fibrillation of denervated muscle fibers, perhaps with some form or degree of peripheral synchronization enhancing mechanical effectiveness. Denny-Brown and Pennybacker (1938) have attempted to answer this question by distinguishing between "fibrillation" and "fasciculation" in muscle, but in view of the other work just described, in which the fibrillation was evidently quite gross, uncertainty exists if this primarily quantitative distinction is basic, if more critical procedures such as nerve block are not required.

The fibrillation begins on the third day in the rat's gastrocnemius (Hines and Knowlton, 1933), and on the fifth at the earliest in the cat's (Langley and Kato, 1915a). It is most conspicuous a few days after onset and weakens thereafter as the muscle wastes, but in every case reported it has continued for the duration of observation unless reinnervation of the muscle supervened, in Schiff's experiments for three to four months, and in some unpublished studies of my own for one year. Just how long fibrillation can continue is a matter for speculation only, but probably so long as the muscle survives as a contractile tissue. In this connection an observation made by Proebster (1928) may be pertinent. The case was one of congenital paralysis in a thirteen year old boy, probably from traumatic birth injury; the left biceps had $\frac{1}{10}$ normal strength and the right, $\frac{2}{3}$; the left biceps, when at rest, showed continuous, small, irregular action currents of a frequency around 110σ; neither the right biceps nor normal muscle showed these, but other cases of longstanding postpoliomyelitic paralysis did. This intramuscular process produced no fibrillation visible through the skin, nor other overt action, and no subjective sensation. Although Proebster considered that these action potentials in apparently quiet muscle must represent a periodic discharge from the central nervous system, in the absence of decisive evidence such as might have been obtained by producing a

temporary nerve block to the muscles in question, it is equally possible that they represented the electrical counterpart of the fibrillation of denervated muscle. In form, the potentials differed in no respect from the potentials led off from fibrillating muscle in experimental animals.

In the experimental animal denervation fibrillation is continuous. The electrical activity (Schäffer and Licht, 1926; Brown, 1937; Rosenblueth and Luco, 1937; Denny-Brown and Pennybacker, 1938) consists in small, asynchronous, diphasic potentials. Determination of rate is tied up with determination of the unit in action, and with possibilities of pacemaking and synchronization. From his electrical records Brown put the rate at 2 to 7 per second, with the single muscle fiber as the unit. This is not far from the rate in Proebster's case, 9 a second. In one cat examined by me (unpublished) an apparent rate of this order persisted unmodified throughout a year of observation, amplitude only diminishing as the muscle atrophied. Langley and Kato (1915a) and Langley and Hashimoto (1918) observed that rate and intensity differed in different muscles, and in different parts of one muscle, but that the rhythm was regular in any one spot. They described the contraction as exceedingly localized, 0.5 to 1 mm. long, as if it occurred in a small region only, of one fiber and spread out very little from this region. My experience confirms this, as against Brown's and Denny-Brown and Pennybacker's opinion that the unit in action is the entire muscle fiber. If the contraction does indeed involve only a small part of the fiber, and the adjacent, uncontracted parts of the fiber are at the same time stretched so that the contraction creates no tension between the origin and insertion, then the failure of this slight continuous activity in the muscle to create tone is accounted for. Such stretching of parts of muscle fibers adjacent to the region in contraction may quite reasonably be expected to contribute to the damaging effect of denervation, particularly in conjunction with any exterior strain which would take up the protective slack.

Irreversible contracture: Following denervation, muscle gradually develops a set or non-ductile condition which is called contracture. This is insidious in onset, and in my experience delayed for from six to eight weeks. It then appears as fixation of the flexor muscles of an extremity in a shortened state, which increases in rigidity for some months. The antagonists of the shortened muscles, in this case the extensors, should correspondingly be fixed in an extended state, but do not seem to have been examined. When fully established, the contracture is not relaxed by general anesthesia, nor by shutting off the blood supply with an

Esmarch bandage, nor in death. Whether in its insidious onset it is equally irreversible is not known. The contracture is, however, only relatively irreversible. Reinnervation of a muscle will, in my experience, partially undo even a fairly high grade contracture of some months' duration, the limitation being set probably by the fibrous tissue present. At the other extreme, with the rapid, massive destruction of muscle tissue which is seen in some badly handled cases of poliomyelitis in man, particularly in muscles such as the deltoid and the anterior tibial group which are subject to gravity strain, contracture is said never to develop. Disappearance of contracture has also been described as the last stage of posthemiplegic atrophy in man, by Brissaud (1880). If contracture depends upon the survival of muscle tissue in some form or to some extent, as these last observations indicate, then contracture must represent a change in the physiological properties of denervated muscle, but physiological study of the contractile mechanism has not extended into this period. Apparently all parts of the muscle do not go into contracture simultaneously, since fibrillation continues at least to the end of a year, and a feeble response to constant current remains for perhaps many years.

Morphological observations. The outstanding morphological result of total denervation of muscle is atrophy, which reduces the gross bulk of the muscle, and the dimensions of each of its constituent fibers. Following atrophy is degeneration, which destroys large numbers of the fibers, although rarely if ever all. Considered morphologically, the processes of atrophy and degeneration, though consecutive, are, by definition, distinguishable; for atrophy reduces the size of a cell, without destroying its form, whereas degeneration either eliminates the cell completely, or so alters its form that its specific differentiation is no longer recognizable. In older literature, these two processes were often referred to as simple and degenerative atrophy, respectively. An outline of the morphological changes taking place in mammalian skeletal muscle during the first few months after denervation was sketched by Mantegazza in 1865, and filled in by Ricker and Ellenbeck in 1899 in substantially the form which holds today. The later stages up to one year were first examined by me (1935). Since most human material is complicated either by incompleteness of denervation, as in postpoliomyelitic cases, or as in traumatic cases, by regeneration, morphological study of the reaction of muscle to denervation has resorted almost entirely to experiment on animals.

Gross changes: Gross loss of bulk or weight begins to be apparent on

the third day after denervation (Langley and Kato, 1915b; Knowlton and Hines, 1936), proceeds most rapidly during the next few weeks, and then slows down. Values of 79 per cent weight loss have been reported by Knowlton and Hines for the rat's gastrocnemius forty-two days after denervation, and of 88 per cent by Chor, Dolkart and Davenport (1937) for the monkey's gastrocnemius-soleus group after twelve weeks. These are, however, the extreme; usually weight loss proceeds more slowly. Since Knowlton and Hines estimate that from 10 to 20 per cent of the original muscle weight is connective tissue, blood vessels, etc., these extreme figures should represent very nearly complete destruction of the muscle tissue.

Besides losing mass, denervated muscle also changes color and texture. According to Langley and Hashimoto (1918), denervated white muscle takes on a pinker tinge, and denervated red muscle becomes less dark red. The mixed muscles of the human and other animals become yellowish. Morpurgo (1892), and Ricker and Ellenbeck (1899) both stressed an early edema in the muscle affecting its texture, but the gravest change in texture comes later with the loss of ductility in contracture. Langley (1916) reported that the specific gravity of denervated muscle is less than normal.

Microscopic changes: The sole-plate: Microscopically, the earliest reaction of muscle to denervation appears in the sole-plates. Boeke (1916) confirmed Gessler (1883) in describing there, in conjunction with swelling and disintegration of the nerve tissue, specific reactions on the part of the two recognized groups of sole-plate nuclei whereby those close to the nerve tissue, the "Telodendrienkerne" atrophy and disappear, while the outlying nuclei, the "Sohlenkerne", increase in number amitotically, and in length. Tello (1907a) did not agree with this. The granular sole-plate cytoplasm survived in Boeke's experiments until regeneration took place; in Gessler's, for sixty days. I found that when the cat's interosseous muscle was kept denervated for one year, the sole-plate cytoplasm diminished rapidly from the second month onward, disappearing completely by six months. Since in these late stages sole-plate and subsarcolemmal nuclei can no longer be distinguished, after six months neither sole-plates as such nor the sites they originally occupied were recognizable. Pommé and Noël (1934) have further described diminution to disappearance of the sole-plate granules called by them telosomes in assorted neuro-muscular disorders in man, most significantly in progressive muscular atrophy, but these granules seem never to have been examined after experimental denervation.

Subsarcolemmal nuclei: Within the body of the muscle fiber, nuclear changes have been reported as early as the second day (Schmidtmann, 1916), and from then on they progress rapidly. By the second week of denervation the subsarcolemmal nuclei have rounded up or become oval, and are dispersed through the fiber; their chromatin is depleted; their nucleoli are reduced to one or none; and their nucleoplasm has lost its homogeneous quality. Toward the end of the first month, the nuclei have become definitely vesicular, sometimes bloated to an extraordinary degree, and fragmenting specimens may be found.

Equally conspicuous with the change in form of the nuclei is the rapid, large, apparent increase in their number, but whether or not this apparent increase is also real, is controversial. On inspection, the clusters and chains of nuclei, in some places packed in the slim sarcolemma sheath as closely as in the equatorial region of a spindle, leave no doubt in one's mind. But when Willard and Grau (1924) made counts of the numbers of nuclei and of fibers present in specimens of mouse muscle from 3 to 63 days after sciatic section, they could find no change in either. Unquestionably a large part of the apparent increase can be, and is achieved in the course of rapid atrophy, by condensation of the nuclei originally present into a progressively smaller volume. And a small part may be due to the greater conspicuousness of the nuclei, bloated and scattered through the fibers. Division, if it takes place, must be amitotic, but the actual division has not been observed, although the converse process of nuclear conjunction has. Stier (1896) studied the muscle of dogs and rabbits for 3½ months after denervation, and came to the conclusion that although the increase of nuclei is largely apparent, due to crowding, both division and destruction also take place so that absolute numbers may be increased or decreased, or presumably not altered at all.

After four to six months and on to the end of a year the nuclei resume their original elongated form and orientation parallel to the length of the fiber, but not their original subsarcolemmal location. Those surviving to the end of this time, are excessively slim. In these late stages also, nuclear destruction must proceed slowly but fairly steadily, for in my specimens taken a year after denervation, nuclei are no more numerous, field for field, than in specimens taken after two months, yet the total bulk of the muscle, in this study always the entirety of an interosseous muscle, had undergone considerable reduction in the interval.

Cytoplasm: Obviously the process of muscular atrophy attacks the cytoplasm of the muscle fiber. But what exactly happens to the cyto-

plasm in the rapid reduction of denervation atrophy is not agreed on, and in this disagreement preconceived ideas of the structure of the cytoplasm seem to be the determining factor. Although the contrary view has had proponents, the opinion of the majority has held that atrophy attacks the sarcoplasm first, and the myofibrillar substance later. This *proposition, which has been defended by me (1935) among others,* assumes that sarcoplasm and myofibrils are discrete. Certainly there is a non-striated cytoplasm in the sole-plate, and filling in around the nuclei. Moreover, fibrils develop in the embryo, or in tissue culture as individuals in a non-fibrillar medium. But that any such duality of structure persists throughout the normal, fully developed, striated muscle fiber of mammals, is very questionable. Hürthle and Wachholder (1925), reviewing the histological structure and optical properties of muscle for Bethe's Handbuch, came to the conclusion (p. 114) "Das Sarkoplasma ist also mehr eine schematische Abstraktion als ein histologischdarstellbares Gebilde". The vulnerability of the sarcoplasm in the process of atrophy appears to derive primarily from its obscurity. Indeed, for the first month or two after denervation, more non-fibrillar material is to be seen around the clumps of bloated nuclei than in any corresponding, well fixed normal muscle fiber.

All through the early stages of atrophy, and in my experience to the end of a year, the cross and longitudinal striated structure of the fiber survives in remarkably good order. In the very first weeks, to be sure, the longitudinal fibrillae may appear loose and disarranged (Ricker and Ellenbeck, 1899; Chor, Dolkart and Davenport, 1937). But thereafter, except when attacked by specific degenerative processes to be described later, the alignment of cross and longitudinal striation is not disturbed, and for some months, at least, the long dimensions of the component parts of the sarcomere are normal for any given state of contraction (Ricker and Ellenbeck, 1899; Tower, 1935). After a month or two the cross striation is clearly faded, becoming finally scarcely more than a shadow; but even in this extreme state alignment is maintained. And the ability to contract in the usual form during fixation survives in some fibers, although from six months on the majority of fibers are composed of very long sarcomeres in which the isotrophic segment I is longer than the anisotrophic A, but in which Hensen's stripe is formed.

Obviously, however, by the time atrophy has progressed to the point of a 50 per cent to 80 per cent reduction in mass, the fibrillar structure must be gravely depleted, yet there is no discontinuity in the processes observed to indicate a shift of attack from sarcoplasm to fibrils. Re-

increase in the fibrous connective tissue of denervated muscle. My specimens of cat's interosseous muscle, taken after two weeks to one year of maintained denervation by unvarying procedure, showed for the first 4 months a fairly uniform condition of half to full contraction of the sarcomeres, but from 6 months on shortening was less and less in evidence, with sarcomeres up to 4 μ in length, or longer, but I longer than A. Yet during this time the muscle was becoming increasingly involved in contracture. This suggests that in contracture the sarcomere is in contraction, but stretched (Buchthal, Knappeis and Lindhard, 1936). Since the interosseous muscles were grossly shortened, yet there was no lengthwise crowding of the content of the muscle fiber, the number of sarcomeres in a given length of such muscle fibers must have been reduced. Again, this presents a difficulty if one conceives of the fibril as existing preformed, but not if one conceives of the muscle fiber as a continuum.

In the irreversible contracture of localized tetanus Davenport, Ranson and Stevens (1929) have similarly described the sarcomeres as of ordinary length, or unusually long in the I component, which they, too, accept as evidence of stretching.

Chemical Observations. There is no single outstanding chemical result of denervating skeletal muscle, but rather, two groups of results relating respectively to fibrillation and to atrophy. Chemical studies of denervated muscle have been more limited in time-scope even than physiological studies. With rare exceptions, they do not begin until the muscle is already in fibrillation, and in no case have they been carried beyond the fourth month. The data in this field are necessarily derived almost entirely from experiment on animals. Rumpf and Schumm (1901) have made one fairly comprehensive examination of a case of alcoholic polyneuritis in man, with reaction of degeneration in the muscles investigated, the results of which agree remarkably with those obtained from animals considering that the material was obtained 3 hours after death and the control values from the literature.

Water content: A number of studies have been made of the water content of denervated muscle, employing simple procedures such as drying in an oven at from 100° to 106°C., or desiccation at reduced vapor pressure, without greatly differing results. Chor, Dolkart and Davenport (1937) could find no significant difference between normal and denervated monkey's muscle up to 10 weeks after nerve section, which is in agreement with Chen, Meek and Bradley's (1924) results on rabbits up to 9 weeks, and Westenbrink and Krabbe's (1936) on cats after 3

weeks. Fenn (1937) found the water content of denervated rat's muscle uniformly increased during the first week after nerve section, and ascribed it to increased filtration from the capillaries due to vasodilatation. Remembering the initial edema described by histologists, this may well be an initial factor, although the progressive increase in water from the 1st to the 7th days suggests that other factors also come into effect. Hines and Knowlton (1933, 1937), have made the most thoughtful study of water content. Although their analyses showed an increase beginning 3 days after nerve section, their estimates of the distribution of water between muscle cell and non-muscle cell phases showed a decreased amount of muscle-cell and an increased amount of non-muscle cell water paralleling the decrease in muscle tissue and relative increase in connective tissue such that the atrophic muscle fibers themselves retained a normal water content. All these slightly differing results probably indicate that in the process of denervation atrophy the water content of the muscle tissue proper is not significantly altered.

Electrolytes: Hines and Knowlton (1933, 1937) followed the potassium, calcium, and chloride of muscle during the first 28 days of denervation atrophy in the rat. Of these substances, potassium is associated almost entirely with the muscle fiber, whereas chloride is associated exclusively, and calcium probably largely with the interstitial tissue. *Calcium* showed the most startling change in concentration, mounting steadily until by 28 days after nerve section it was double, or slightly more than double that of the control. This increase may be accounted for in small part by the absolute increase in connective tissue of the muscle and the even greater relative increase of interstitial tissue over muscle tissue, but for the larger part there is no explanation unless it is associated with an increase in inorganic phosphorus to be described later. *Chloride* concentration also increased steadily as atrophy progressed, but only to a degree easily accounted for by the relative increase in non-muscle tissue over muscle tissue. Perfusion experiments showed that the denervated muscle cells were normally impermeable to chloride. *Potassium,* on the other hand, decreased in concentration to an extent entirely commensurate with the decrease of muscle tissue relative to interstitial tissue. Leulier, Bernard and Richard (1932) had previously obtained a similar result.

Fenn (1937) questioned Hines and Knowlton's figure for potassium in the first week, claiming, also for the rat, a slight increase. The significant feature of Fenn's report is, however, the indication, afforded by

his table, of a critical time after denervation for reversal of potassium increment to potassium decrement. In the semimembranosus and tibialis muscles this took place on the 3rd day, the day of probable onset of fibrillation, although in the gastrocnemius and biceps femoris it was delayed to the end of the first week, or perhaps merely obscured by a factor so simple as the one he suggests, namely, depletion of the potassium of the control muscle by excessive activity in compensation for the paralysis. Such a reversal of gain to loss in conjunction with the onset of fibrillation in the previously inactivated muscle is exactly what would be expected from Fenn's results on the depleting effect of exercise on the potassium of normal muscle.

pH: Determinations by Chen, Meek and Bradley (1924) of the pH of denervated and control muscle showed no measurable increase in acidity in muscle undergoing atrophy up to 9 weeks after nerve section. However, the muscle lost a small amount of buffer capacity against both acid and alkali, probably because of an alteration in protein.

Lipoids: Because of the twofold representation of lipoids in muscle, on the one hand as integral and essential constituents of the muscle cell, and on the other as products stored either in the muscle cell or in the interstitial tissue, figures on the lipoid content of muscle are peculiarly liable to variation. For the stored lipoid, especially that stored in the interstitial tissue, will vary with general conditions of nutrition and activity, whereas the structural lipoid of the muscle will change only with changes in muscular state or activity. Hence weak or paralyzed muscles may become heavily infiltrated with interstitial fat in well nourished individuals at the same time that destruction of the muscle tissue must be reducing the structural lipoid. A further complication is introduced, moreover, by the apparent fact that mobilization of fat from fat deposits is gravely impeded by denervation of the region, while deposition is not (Mansfeld and Müller, 1913; Beznók and Hasch, 1937; Cedrangolo, 1937). Figures of total lipoid in denervated muscle are, therefore, of uncertain significance. What appear to be absolute increases in the fat content have been reported by Grund (1912) and Audova (1923), while Westenbrink and Krabbe (1936) could find no significant change. Chor, Dolkart and Davenport (1937) found a percentage increase in the lipoid of denervated monkey's muscle without change in the total lipoid. Obviously any large accumulation of interstitial fat must be taken into account in considering concentration of other substances in the muscle. This was done in the studies of Grund (1912), Audova (1923) and Cahn (1927), but has not been general practice.

Since the stored lipoid is chiefly simple, and the structural lipoid, compound, analyses which discriminate between these might contribute to understanding the processes involved in denervation atrophy. Grund (1912) has reported that the phosphatide-phosphorus content of dog's muscle denervated for 3 months was low if calculated relative to total dry weight, but if calculated relative to fat free dry weight it was the same as in the control. Cahn (1927) found it reduced even in the latter case. Clearly, this work needs to be repeated. Preservation of the phospholipid is not consonant with Bloor's (1937) concept that phospholipid content parallels the functional state of tissues in general, including muscle, increasing in hypertrophy (with reservations and exceptions) and diminishing in atrophy. Bloor did not, however, investigate denervation atrophy in which, because of the fibrillation, the muscle is far from inactive. Cholesterol has been determined only by Westenbrink and Krabbe (1936), who considered it not significantly altered three weeks after denervation in the cat.

Phosphorus: Grund (1912), Avellone (1927), Cahn (1927) and Chor, Dolcart and Davenport (1937) all report a lower total phosphorus content in denervated muscle than in its control. Since total phosphorus is compounded of phosphorus of diverse sorts, it is uncertain what if any significance this has. Certain of the phosphorus fractions are, however, of patent significance. The significance of the lipid fraction has just been considered.

Hines and Knowlton (1933, 1937) found the non-lipoidal, acid-soluble phosphorus of rat's muscle reduced to 60 per cent of the control value after 28 days' denervation. Since this phosphorus is chiefly associated with the muscle cells, and the decrease paralleled the rate of atrophy, acid-soluble phosphorus was probably still present in the atrophic muscle fibers in normal concentration. Acid-soluble phosphorus is, however, still a composite figure. On fractionating the acid-soluble phosphorus Hines and Knowlton (1933) found that the inorganic phosphorus content was slightly increased, and phosphocreatine sharply diminished, down to 30 per cent of the control value in 28 days, while adenosine-triphosphate and the undetermined phosphate fraction were diminished to 50 per cent in a similar time. The possible relation of the increase in inorganic phosphate to the large increase in calcium, has already been mentioned. The loss in the adenosine-triphosphate and undetermined phosphate fraction was, they consider, commensurate with the loss of muscle tissue. On the other hand, the depletion of phosphocreatine was early and rapid, finding a parallel only in the depletion of glycogen to be described later. Westenbrink and Krabbe (1936)

likewise found the creatine-phosphoric acid and orthophosphoric acid fractions of acid-soluble phosphorus reduced out of proportion to the other fractions in denervation atrophy.

An earlier study by Zanghi (1928) permits correlation of this phosphocreatine depletion, and of phosphoric acid accumulation, with the onset of fibrillation. For the first 24 hours after sciatic section in rats, the phosphocreatine of the denervated muscle exceeded the control values by 50 per cent, with phosphoric acid correspondingly low. Even at 72 hours phosphocreatine was still in excess, but phosphoric acid had begun to rise. Thereafter phosphocreatine was rapidly depleted, down to nothing in one case, while phosphoric acid accumulated, and this continued until 45 to 90 days after operation when regeneration probably took place. Although Zanghi seems to have been unaware of the phenomenon of fibrillation, the time correlation is obvious between reversal of relative values in denervated and control muscle on the third postoperative day, and the onset of fibrillation in the rat.

Creatine: Cathcart, Henderson and Paton (1918) made the first extensive examination of creatine content of denervated muscle. Using cats and rabbits, they reported no significant change in creatine content up to the 11th day, but after the 15th day a progressive decrease. Their figures show, however, creatine values considerably higher than the control in two examinations made on the 3rd day and one on the 7th, which raises the question of a possible relationship of onset of creatine loss to onset of fibrillation. Working on rats, Hines and Knowlton (1933) described a beginning loss of creatine on the 3rd day after nerve section, that is, on the day of onset of fibrillation in this species, but gave no figures before this time. By the end of the first week creatine content was definitely lower than in the control muscle, but only to an extent which they consider accounted for by the altered ratio of muscle tissue to connective tissue. Avellone and di Macco (1925) have contributed evidence of similar character from dogs.

Nitrogen: The total nitrogen content of muscle is probably unaltered in the course of denervation atrophy, at least in the earlier stages. Hines and Knowlton (1933) working on rats, Westenbrink and Krabbe (1936) on cats, and Chor, Dolkart and Davenport (1937) on monkeys, all agree on this. Previously, both Grund (1912) and Audova (1923) had found the same to hold for the fat-free dry substance of denervated dog and rabbit muscle. Avellone and di Macco (1925) are alone in reporting a steady decrease.

Setting aside Steyrer's (1904) early attempt to discriminate between

different proteins in the course of denervation atrophy, Hines and Knowlton (1933) determined separately: total extractable nitrogen, the nitrogen extractable by 5 per cent trichloracetic acid which is taken to be non-protein nitrogen, and various nitrogen fractions extractable with different strengths of phosphate solution which may or may not represent true individual proteins. All these maintained a practically constant percentage throughout 28 days of denervation atrophy. One nitrogen fraction which would be of particular significance in relation to the disagreement over nuclear proliferation, would be purine nitrogen. This has been determined only by Avellone and di Macco (1925), who found it sharply decreased, and to the same extent as total nitrogen, after 60 days' denervation,—minus 30 per cent. Since their total nitrogen figure is unconfirmed by general experience, their purine nitrogen figure must be suspect until substantiated.

Hines and Knowlton bring out an important consideration applicable to blanket determinations such as total nitrogen or total phosphorus: that a normal figure for these does not necessarily mean that no changes have taken place in the substances making up the total. In the case of non-protein nitrogen, two substances contributing to the total, namely, creatine and adenosine-triphosphate are known to be lowered in concentration by denervation atrophy. Since other unidentified nitrogenous substances must be correspondingly increased to make up an unaltered total, the changes in chemical composition of the tissue during denervation atrophy must be considerable.

Further evidence that the nitrogenous constituents of muscle are altered in the processes of denervation atrophy has been contributed by Chen, Meek and Bradley (1924). In postmortem autolysis they found that denervated muscle liberates more amino-acid than normal muscle by as much as 100 per cent, and specimens taken nine weeks after nerve section showed this increase equally with specimens obtained one or two weeks after operation. They consider that this shows a gradual, progressive alteration of structural protein into more digestible forms, and suggests an equilibrium in muscle between non-available and available cell protein. Furthermore, they found that although atrophic muscle contains, when removed, no more free amino-acid than normal, it does contain more primary cleavage products as shown by the reaction for tyrosine peptide.

Glycogen and lactic acid: Glycogen analyses of denervated muscle may be classified into: those showing an increased glycogen content, which include all except one of the old reports (Chandelon, 1876; Manché,

1889; Vay, 1894) and two more recent ones (Avellone, 1927; Cedrangolo, 1937); those showing no consistent alteration (Krauss, 1888; Westenbrink and Krabbe, 1936), and those showing a loss (Hines and Knowlton, 1933, 1935; Knowlton and Hines, 1934). Hines and Knowlton alone describe the use of currently accepted precautions in killing the animals and taking the specimens for analysis. For the first 24 hours after nerve section the muscle showed no depletion of glycogen and no failure to store glycogen on glucose administration, but after 48 hours the loss was detectable. During the third day the depletion of glycogen was rapid, amounting on an average to 43 per cent of the control value, after which further reduction was slow.

Results obtained on lactic acid reverse those obtained with glycogen. Avellone (1927) reported a low lactic acid content in denervated muscle compared with the control, and Westenbrink and Krabbe (1936), no significant difference. Knowlton and Hines (1934) found a consistently higher content of lactic acid after 3 to 7 days' denervation than in the control. This increase went hand in hand with depletion of the glycogen store, and both were ascribed by these authors to the fibrillation which begins in rat's muscle on the third day after denervation, which is not abolished by the amytal anesthesia employed in killing the animals. In studies in which a general anesthetic was not properly employed to kill the animals this fibrillary activity in the denervated muscles would be matched against voluntary and reflex activity in the normally innervated muscles with unpredictable results in the balance on the two sides of glycogen and lactic acid.

Summarizing these observations: The chemical materials of denervated muscle in so far as these have been studied, may be considered in five categories. These are: 1, general protoplasmic constituents, showing no change; 2, substances related particularly to the muscle fiber and reduced in proportion to its atrophy; 3, substances found particularly in the interstitial tissues, and increasing with the relative and absolute increase in these tissues; 4, substances probably especially related to interstitial tissues but increasing disproportionately; 5, substances found especially in the muscle tissue, but disproportionately reduced in conjunction with fibrillation. In the first category are total nitrogen and extractable nitrogen fractions possibly representing specific proteins, and water. In the second category are potassium, acid-soluble phosphorus, creatine, adenosine-triphosphate, and perhaps phospholipid. In the third category are chloride and inorganic phosphate. In the fourth category is calcium, and simple fat might be added in individual cases. In the

fifth category are glycogen and phosphocreatine. Lactic acid and phosphoric acid constitute a sixth category of substances produced in the fibrillary activity. Calcium is the only substance requiring further consideration. It is not known how the extra calcium is stored in the muscle. If stored as tricalcium phosphate in the interstitial tissue, or perhaps as carbonate,—carbonates have not been examined,—it may be fairly unreactive and without much significance. On the other hand, if it is available at the surface of the muscle fiber it may contribute to altered conditions there of great consequence.

Respiratory metabolism: Langley and Itagaki (1917) opened the study of the respiratory metabolism of denervated muscle by comparing the oxygen content of arterial and venous blood going to and coming from denervated and innervated extremities of anesthetized cats. They found the oxygen usage of the denervated muscle per unit weight nearly 3 times as great as of the control, and ascribed it to the fibrillary activity in the former. Hines, Leese and Knowlton (1931) have made a more extensive study on anesthetized dogs, comparing for denervated and control muscle: the sugar, lactic acid, oxygen and carbon dioxide content of blood entering and leaving the extremities. The determinations were made during rest, during mild exercise by direct electrical stimulation, after glucose administration and after subcutaneous adrenaline. They found the respiratory quotient of the denervated leg essentially the same as that of the control, and of the entire animal, and likewise the exchange of sugar and lactic acid between tissues and blood; and concluded that muscle undergoing atrophy of denervation exhibits a metabolism qualitatively similar to that of normal muscle as far as can be determined by analysis of the blood. Sato and Kasugai (1935) have contributed evidence of similar character.

In a later study Knowlton and Hines (1934) took up the quantitative analysis of the respiratory metabolism of excised specimens of rat's gastrocnemius muscle denervated 3 to 28 days. Spontaneous fibrillation ceased shortly after these specimens were excised. Under these conditions both the rate of oxygen consumption and the amount of glycogen disappearing and of lactic acid appearing during 30 minutes of anaerobic autolysis were much the same in denervated and control muscle. However, considering that the process of atrophy increases the relative and absolute amount of connective tissue in the muscle, and that connective tissue has a lower rate of oxygen consumption than muscle, a rate of oxygen consumption for the denervated specimen as a whole equal to that of the control must, they believe, represent a slightly increased

rate for the denervated muscle tissue itself. In the light of this careful study, Califano's (1927) earlier report of a greatly increased in-vitro O_2 consumption by denervated muscle must be viewed with scepticism.

Succino-dehydrase activity: Knowlton and Hines (1934) also examined the extractable succino-dehydrase activity of denervated muscle. In contrast with the apparent viability of the hydrolytic cleavage system responsible for glycogenolysis and lactic acid formation, this activity fell off to about 55 per cent of the control value within one week after denervation.

Glutathione: Okuda (1930) reported that 3 days after section of the sciatic nerve, before the muscle had lost weight appreciably, reduced glutathione was present in greater amount in denervated rabbit's muscle than in its control by 4 to 32 per cent. By 20 days after nerve section, when the difference in weight was marked, glutathione ranged from 30 to 167 per cent higher in the atrophic muscle. This, he believed, reflected the increased metabolism of the denervated muscle.

Respiratory pigments: Of the two pigments present in muscle, cytochrome and muscle hemoglobin, the generally distributed cytochrome has not been examined in relation to denervation atrophy, while the hemoglobin, which is found only in red muscle, has. Whipple (1926) reported a rapid reduction in the hemoglobin of dog's gastrocnemius muscle undergoing denervation atrophy, amounting to 50 per cent of the control value. A large part of this loss can, however, be accounted for by the increase of connective tissue relative to muscle tissue, so that within the muscle cell the concentration would not have been so greatly altered. An interesting problem, as yet uninvestigated, is presented by the change in color of atrophying muscle, both white and red becoming pink. If this represents acquisition of hemoglobin by the white fibers and loss by the red, hemoglobin values for the muscle as a whole should be a function of the relative proportions of red and white fiber originally present.

Summarizing these observations: The data on respiratory metabolism of denervated muscle deal with two almost distinct problems. On the one hand, comparative figures for denervated and control muscle on oxygen consumption in-vivo, on content of glycogen, lactic acid and phosphoric acid, and probably also of glutathione, measure the difference in metabolism between the continuously fibrillating denervated muscle and the resting metabolism of the control, the animals being anesthetized. They show that fibrillation involves a high grade oxidative metabolism which does not differ qualitatively from the metabolism of

normal muscle, quantitative comparison being impossible without figures on heat produced or work done. Similar figures from the later stage of contracture are unfortunately not available to substantiate the suspected non-oxidative basis of this condition. On the other hand, the in-vitro studies examine the resting or survival metabolism, and the basic metabolic capacities of the denervated and control tissue. These studies indicate that the denervated muscle fiber probably has a slightly increased oxidative metabolism. Knowlton and Hines (1934) suggested that this is due to the relative increase in the number of nuclei. Consideration might also be given to the possible effect of altered polarization of the cell surface on the resting O_2 consumption (Fenn, 1931), as well as to the effect of the increase in surface area of muscle fibers per unit mass of tissue resulting from the atrophic reduction in diameter of the individual fibers. These studies have furthermore shown that the mechanism for glycogenolysis and lactic acid formation remains intact during at least the first week of denervation atrophy, whereas the oxidative system represented in the extractable succino-dehydrase activity is rapidly impaired during the same interval. Whether or not the muscle glycogenolytic system survives unaltered in later stages is not known, but probably not, for even in this first week an increased sensitivity of the system to thyroxin (Hines and Knowlton, 1935) represents some departure from the normal.

ACCESSORY FACTORS IN THE REACTION OF MUSCLE TO DENERVATION. In the preceding account the reaction of muscle to denervation has been described chronologically so far as the evidence goes. Qualitatively this is feasible, but not quantitatively, for a variety of factors influence onset, rate of progress and final severity of the processes involved.

The phylogenetic factor is the factor of greatest moment,—setting apart the reactions of warm- and of cold-blooded animals. That denervation atrophy runs in birds a course similar to that in mammals was long ago established by the work of Knoll and Hauer (1892) on the dove. In amphibians, on the contrary, Scaffidi (1913), Adrian and Owen (1921), Watts (1924) and Bremer and Gérard (1925) all failed to find the changes in excitability and in contraction in denervated muscle familiar in the mammal. And Langley (1916) reported that denervated amphibian muscle does not fibrillate. Bremer and Gérard ruled out the factor of low environmental and body temperature in this connection. However, cold-blooded muscle cannot be exempt from all reaction to denervation, for there is too much morphological and chemical evidence to the contrary, including a bit of Langley's (1916) own. Furthermore,

although the reaction is unquestionably delayed in onset and perhaps lacking in important features, most notably fibrillation, it is none the less basically atrophic. In the absence of fibrillation, this may be atrophy of disuse.

In a stimulating paper, Knowlton and Hines (1936) have submitted evidence of a species difference in rate of denervation atrophy, and even of individual differences in a genetically non-homogeneous group such as dogs, contrasted with uniformity in genetically homogeneous groups such as rats, mice, guinea pigs and pigeons. This species-characteristic rate of atrophy they relate to growth rate and to life span, a short life span being associated with a rapid rate of denervation atrophy.

Stier (1896) recognized an age factor by which denervation atrophy progresses more rapidly in young animals than in old, but this needs more careful evaluation.

The kind of muscle under examination, whether red or white, thin fibered or thick, may also make a difference to the course of the reaction to denervation. On the whole it appears probable that white muscle wastes more rapidly than red, and certainly fibrillation is particularly strong and rapid in the white gastrocnemius (Langley and Kato, 1915a; Langley and Hashimoto, 1918). Morphologically, denervation atrophy abolishes the distinction between the two kinds of muscle. The gross evidence for this has already been given. Knoll and Hauer (1892) have made the outstanding microscopical report to the same effect on the breast muscle of the dove. Here the thick fibers diminished in size much faster than the thin after denervation so that after 35 days there was no longer any distinction of fiber types on this ground. During the same time the granules which appear in the thin fibers in specimens removed from the body and studied fresh, rapidly ceased to appear thus eliminating this morphological distinction also.

Certain other factors which might influence the rate of atrophy of denervated muscle have been investigated by Hines and Knowlton (1934a, b). The denervated muscles of fasting animals lost weight at a faster rate than the rate due either to fasting, or to denervation alone, but the total loss was less than the sum of the losses suffered by the fasting control muscle and the non-fasting denervated muscle. Thyroxin administration accelerated the rate of denervation atrophy, whereas thyro-parathyroidectomy preceding nerve section slowed it. Environmental temperature had no influence. Likewise the administration of 0.2 gram of glycine per day per rat was without effect in the

first week. Richter (1935) has confirmed this last with administration over a longer period of time.

A factor which might influence rate of onset, though not final severity of the reaction of muscle to denervation is the distance between nerve lesion and muscle. Adrian's (1917) comment that the facial case he reported in detail had the fastest rising chronaxie of his experience brings this to mind, in conjunction with the rapidity of developments in the rat's gastroncemius. Such a factor, if demonstrable, might be related to the controversial question of centrifugal versus centripetal versus coincident degeneration throughout the cut-off length of nerve.

In comparing the work of different observers, variables of procedure must enter in. Trauma, ulceration and secondary infection have already been mentioned as accelerating factors in the reaction to denervation. State of nutrition may also have a very material influence, even beyond that brought out in consideration of the lipoids of muscle. As a retarding factor there is regeneration, which must be suspected in all long continued studies of the reaction, and if possible restrained by repeated operation.

Present day treatment of paralyzed muscle, particularly in post-poliomyelitis cases, takes account of other traumatic factors contributing to the destruction of muscle by putting the paralyzed extremities at absolute rest and relieving all stress and strain, especially the strains imposed by gravity acting on dependent parts and by non-paralyzed muscles acting against paralyzed antagonists. Experimental work has largely neglected these factors, and selected as study object in the majority of cases the gastrocnemius muscle, a muscle peculiarly liable to just such trauma. It is not surprising, therefore, that except for Knowlton and Hines' results (1936) on the dog, rapid, severe atrophy has characterized results obtained from this muscle (Sherrington, 1894, cat; Ricker and Ellenbeck, 1899, rabbit; Langley and Kato, 1915b, cat; Langley and Hashimoto, 1918, rabbit; Willard and Grau, 1924, mouse; Hines and Knowlton, 1933, rat; Chor, Dolkart and Davenport, 1937, monkey, cat). In striking contrast are my results (1935) on the interosseous muscle of the cat's fore paw: an average weight loss of less than 50 per cent[1] after 4 months, and of only 67 per cent after a year. In this work, the factor of regeneration was excluded by re-operating as often as necessary. But stress and strain were also very nearly ex-

[1] The figure of 50 per cent average loss after four months' denervation should read 46 per cent, making the total average loss 59 per cent instead of 60 per cent.

cluded because the cat either kept the affected extremity raised off the floor by action of the shoulder muscles, later assisted by flexor contracture, or he walked on the dorsum of the paw with the paw and digits turned under. Thus the only tension to which the interosseous muscles were subjected was the very slight dead weight of the toes, and later, possibly, the tension of contracture in the muscles themselves. Roberts (1916) and Kopits (1929) may be cited as authority for the statement that in man stretched, denervated muscle atrophies more gravely than relaxed muscle, although the observation has been very generally made.

At present it is probable that no study yet made is a study of uncomplicated denervation atrophy. The facial musculature, lacking direct bony attachment, little exposed to ordinary injuries, with the dependent weight only of itself and the overlying skin, affords a unique opportunity which has not been taken advantage of, although Duel's (1932) cases of extraordinarily long survival of this muscle in man may be recalled in this connection. Other possible examples of slow atrophy in muscles relatively free of stress and strain are Schiff's (1851) comment that the tongue musculature atrophied more slowly than the muscle of the extremities after denervation; Sherrington's (1897) failure to find clear-cut atrophic changes in the cat's extraocular muscles 60 days after section of the oculomotor nerve; and Arloing and Chantre's (1898) report that the sphincter ani of the dog remained almost intact, anatomically and functionally, one year after bilateral section of the nerves to it. In such cases, however, the possibility that rate of denervation atrophy is characteristic of a given muscle and greatly different in different muscles, as Langley and Hashimoto (1918) believed, must also be given careful consideration. Nevertheless, such differences illustrate the necessity: first of understanding, and then of controlling so far as possible the accessory factors contributing to the reaction to denervation in muscle.

Share of the Components of Peripheral Innervation in the Reaction of Muscle to Denervation. That motor denervation has the major responsibility for the reaction of the extrafusal muscle to total denervation is universally recognized. The only question which needs examination is whether or not it is solely responsible, and if not, which other components are accessory, and in what manner.

Posterior root innervation should contribute to the well-being of skeletal muscle by the protection afforded by intact sensibility against trauma of all sorts, and by the proprioceptive support of muscle tone. Furthermore, the posterior roots present the possibility of efferent action on muscle, either antidromically over the sensory fibers, or, less probably,

over fibers emergent from the cord. When put to the test, however, these appear to be very minor neurotrophic factors.

Mott and Sherrington (1895) reported for monkeys a very moderate wasting of muscle 4 months after posterior root section, without change in color or in electrical reactions. "Trophic sores" were, however, a complication. Working with cats I (1931a) could not detect even this moderate reaction; a year after ablation of the posterior root ganglia supplying the fore-leg the extrafusal muscle fibers showed no morphological change whatever, gross or microscopic. Whether a species difference, or the coincidence of ulceration be invoked to explain the discrepancy between monkey and cat, neither posterior root section by which only fibers emergent from the cord would degenerate, nor ganglionectomy by which all the fibers would be destroyed, produced sufficient effect to give scope to a specific neurotrophic function of the posterior roots. Unfortunately, the skeletal muscle has not been examined in the numerous extensive posterior rhizotomies in man of indefinite survival, but the lack of reports of atrophy in these cases may be taken to indicate that in man as in other mammals the total neurotrophic effect of the posterior roots is negligible.

The work of Kuré (1931) and his associates is not considered in this connection.

Sympathetic denervation: A variety of changes have been described as taking place in muscle after sympathetic denervation: changes in muscle tone and in fatiguability, in metabolism, in glycogen content, in chronaxie. That the sympathetic system plays no special rôle in the maintenance of ordinary tonic contraction, is now adequately proven, but its relation to fatiguability, to metabolism in general and to glycogen content in particular still needs further elucidation. The altered chronaxie (Barron, 1934) is apparent only when muscle is exercised. None of these changes clearly reproduces any part of the reaction of muscle to total denervation. Acceptable visible evidence of atrophy and degeneration would, however, be decisive. At the time of the controversy over the sympathetic innervation of tonic contraction several reports to this effect were made (Hunter, 1925; Kerper, 1928; Gaissinsky and Lewantowsky, 1929). On the other hand, a larger and longer series of investigations (Jonnesco and Floresco, 1902; Floresco, 1903; Takahashi, 1922; Feldberg, 1926; Coates and Tiegs, 1930; Nevin, 1930; Tower, 1931b) has yielded no morphological evidence whatever of atrophy or degeneration in muscle as long as one year after sympathectomy in experimental animals and up to six and a half years in man.

Likewise, sympathetic denervation has had no demonstrable influence on the postnatal growth and development of skeletal muscle (Cannon, 1930; McCullogh, McFadden and Milroy 1930; Harris and Wright, 1930; Tower, 1932a).

Combined posterior root and sympathetic denervation has also been examined for effect on the extrafusal muscle fiber (Tower, 1931a; Hinsey, unpublished), again without result. Hinsey's study gave statistical treatment to measurements of cross sectioned area of over 8,000 muscle fibers, half normal, half affected by the dual operation for a period of 2 years.

Posterior root or sympathetic denervation in conjunction with ventral root denervation: Although separate elimination of the posterior root, and sympathetic innervations of the organ, skeletal muscle, does not evoke any part of the reaction to denervation in the extrafusal muscle tissue, this does not exclude the possibility of their accessory contribution to the reaction to motor denervation. Particularly might the sympathetic innervation be expected to have some influence through its vascular control. To examine these possibilities systematically in cats, I compared (1935): first the effect of ventral root section with that of peripheral nerve section; then the effect of ventral root plus sympathetic ganglionectomy, and of ventral root plus posterior root ganglionectomy with peripheral nerve section, each of 4 months', 6 months' and 1 year's duration. Even including endothelial hypertrophy, all the results of total denervation of muscle were reproduced by ventral root section only, or by ventral root section in combination with dorsal root or sympathetic ganglionectomy, with the exception of certain results in the spindle which will be separately considered next.

The reaction to denervation in skeletal muscle therefore develops entirely as the result of motor denervation, the other innervations of the organ, skeletal muscle, making no contribution whatever.

Reaction to Denervation in the Muscle Spindle. Because of its double innervation, by sensory and by motor nerve fibers, the muscle spindle presents a special, and interesting problem in neurotrophic relationship on which so far only morphological evidence is available. For a long time it was thought, on the authority of Sherrington (1894), that the spindle is exempt from regression following denervation. This is not the case. Even before Sherrington, Onanoff (1890) had presented evidence from man and from experiments on dogs, suggesting that the intrafusal muscle fibers are in trophic and motor dependence on the anterior horn cells. Among later writers, Forster (1894), Horsley

(1897), Tello (1907b), and Willard and Grau (1924) have supported Sherrington, while Batten (1897), Jamin (1904), Boeke (1916), Rosin (1919), Tower (1932b), and Chor, Dolkart and Davenport (1937) have agreed in essence with Onanoff.

The only study of the effects of differential denervation in the spindle is my own (1932) on cats. Taking up first the problem of whether or not the spindle exhibits any reaction to denervation, I examined the condition of the spindle after total denervation of four months' to one year's duration. Denervation was maintained by repeating the peripheral nerve section as often as necessary. By this means it was established that all parts of the spindle react to total denervation in much the same manner and time as the rest of the muscle, although because of the initial small size of the muscle fibers, and the central location of their nuclei, the early and atrophic changes are less conspicuous. The muscle fibers become smaller, the nuclei first round up and later swell and degenerate, the cross striation fades, the longitudinal striation intensifies, and unless acute degenerative processes attack and destroy the intrafusal fibers first, the end is fibrous metaplasia of the muscle fiber. The investing fibrous connective tissue is also increased, and the vascular endothelium thickened.

To determine which of the components of peripheral innervation contribute to the reaction, and what, I then examined the spindles after the components of innervation had been separately eliminated for from four months to one year. Sympathetic denervation produced no noticeable effect in the spindle. With ventral root section, the polar regions of the intrafusal muscle fibers atrophied and degenerated along with the extrafusal fibers, and the capsule thickened, but the equatorial region remained intact. These regions, invested by a dilated and thickened capsule, then formed conspicuous objects in the mass of otherwise atrophied muscle. After dorsal root ganglionectomy, the reaction was confined to the equatorial region. Here the number and volume of nuclei were reduced, seemingly by fusion, while the cross striated substance gradually increased until the intrafusal muscle fibers took on throughout their length the morphology of the polar regions. The capsule, which was not thickened, also invested the fibers more closely.

The dorsal and ventral spinal roots therefore contribute to the reaction to denervation in the muscle spindle as they share its innervation. Following partial denervation, motor or sensory, that portion of the structure of the intrafusal muscle differentiated to receive the destroyed innervation atrophies and degenerates: after motor denervation the

cross striated structure; after sensory denervation, the equatorial aggregation of nuclei. The reaction to motor denervation resembles in all respects that of the extrafusal muscle fiber. The reaction to sensory denervation, on the other hand, introduces new phenomena and new considerations.

Analysis of the Reaction of Muscle to Denervation. Reviewed as a whole, the reaction of muscle to denervation takes place in three phases. First is a period of three to perhaps seven days after nerve section during which the muscle is inactive. In this period the nervous tissue in conjunction with the muscle degenerates. Within the muscle tissue itself, the changes recorded are slight. Morphologically, the nuclei begin to react both in the sole-plates and in the body of the muscle fiber, but atrophy is negligible. Chemical study has not scrutinized this early phase minutely except in Fenn's (1937) potassium analyses made for quite another purpose, but such fragments of evidence as are available are suggestive. Physiologically, the emergence of slow contraction toward the end of this period, and the rising chronaxie demonstrate that far-reaching alterations are taking place, beginning perhaps at the moment of denervation.

Abruptly, the end of the first phase and the beginning of the second are signalled by the onset of spontaneous, rhythmical contraction which then continues probably for so long as the muscle survives, perhaps for years. The development of this activity appears to coincide with the disintegration of the nervous tissue. Certainly it ushers in rapid, progressive atrophy, a process involving reduction in the size of the muscle and of each of its component fibers. Throughout this phase the characterizing structure of the tissue is conserved in the cross and longitudinal striations, together with its characterizing function, contraction, and a normal content, estimated for the muscle tissue itself, of water, protein, phospholipid (?), phosphate and creatine, and potassium,—that is, of the essential materials of chemical structure.

Transition from the second to the third phase is gradual, over months and perhaps years. By atrophic elimination of the substance of the muscle fibers until cross striation is no longer visible and only longitudinally disposed fibrils remain, and by proliferation of interstitial fibrous tissue, a structure is produced which is intermediate between scar tissue and tendon, and almost certainly incapable of either excitation or contraction.

Interrupting the regular sequence of atrophy and transformation into fibrous tissue, acute degenerative processes may lead at any time to

complete destruction of single fibers, or groups of fibers, and their replacement by scar tissue, but these acute degenerative processes appear to be accidentally determined, and not, like atrophy and fibrous metaplasia, the inevitable consequence of denervation.

The mechanism of the reaction of muscle to denervation is less easily analyzed than its course.

The reaction of the nuclei of muscle to denervation, early and late, may be fairly adequately accounted for. The reported degeneration of the central nuclei of the sole-plate presents the greatest difficulty, but the probability that these are non-muscular in origin (Couteaux, 1938a), makes an explanation less imperative in this review, while opening possibilities of specific neurotrophic relationship between nerve and its investing neurilemma. The exterior nuclei of the sole-plate and the subsarcolemmal nuclei react similarly in a manner which suggests initial stimulation, followed either by death or by subsidence of the stimulated state. That the sole-plate nuclei should react is not surprising, considering their proximity to the disintegrating nervous tissue, but the reaction of the nuclei all up and down the sarcolemma, and before the process of nerve disintegration has reached a climax, strains the concept of toxic stimulation by the products of disintegrating nerve. Loss of a specific neurotrophic agency cannot very well be again invoked because later the nuclear reaction subsides, while the absence of such an agency should continue in effect. The early change in form of the nuclei is probably most simply explained by Speidel's (1938) observation that in tadpole muscle fibers relieved from tension, the nuclei round up and develop a single nucleolus. Quite possibly the early rounding up of the nuclei in denervated muscle represents nothing more than a reaction in the initially flaccid muscle to release of tension, with subsequent lengthening out again much later as contracture sets in, establishing a new unremitting structural tension. The original loss of orientation to the sarcolemma, and failure to reorient to it may, however, be evidence of some additional change relating specifically to the cell surface.

The depletion of nuclear chromatin and swelling and disintegration of the nuclei in the first weeks or months suggest fatigue proceeding to exhaustion, which may be ascribed to the continuous functional demand of fibrillation. From the sixth month on, however, the surviving nuclei resume much their original form though not their orientation to the sarcolemma, and thereafter atrophy and disappear commensurate with the cytoplasmic atrophy in a manner suggestive of the maintenance of a nucleo-cytoplasmic ratio such as Schiefferdecker (1909) has postulated.

If there is, in fact, no nuclear proliferation, the totality of the nuclear reaction early and late is thus fairly adequately accounted for. If, on the other hand, there is a real proliferation, this may represent an initial response on the part of the denervated muscle fiber to the increased functional demand of fibrillation whereby the nuclear mass is increased as Schiefferdecker's theory requires. Later, this demand should diminish again, slowly with the atrophic reduction in the mass of cytoplasm, and more rapidly as the muscle goes into irreversible contracture, permitting the later reduction in nuclear mass. The old concept that the nuclear reaction is regenerative is not supported in any way by results produced.

That two modes of excitation and contraction, fast and slow, are present in the normal muscle fiber is now fairly well established. (For evidence and discussion read Bremer, 1932.) These two modes of response need not represent separate contractile mechanisms such as the sarcoplasm and fibrils of Bottazzi's (1901) old concept, but merely two modes of employment of one basic mechanism of muscular contraction by excitatory processes which differ as much as 100 fold. In normal muscle the rapid mode of contraction is dominant. Denervating skeletal muscle brings the slow mode into prominence partly by weakening and probably eliminating the rapid mode, partly by increased excitability of the slow mode as evidenced by the increased sensitivity to electrical stimulation by constant current and to mechanical stimulation, as well as to a variety of chemical and pharmacodynamical agents. Contractility itself survives, to respond in either mode so long apparently as the basic structure survives at all, probably for years, although finally the muscle becomes more or less involved in irreversible contracture. The disappearance of the rapid mode does not correlate with disintegration of the nerve fiber, which is complete in a matter of days, for chronaxie only begins to rise sharply as indirect excitability is abolished. And the rapid contraction is known to persist for at least a month, and probably for longer. This locates the rapid mechanism, and its degeneration, within the muscle fiber. There, the onset and final completion of changes in the quick mode of excitation and response parallel suggestively the reaction and destruction of the sole-plates. The degeneration of the central nuclei and associated cytoplasm may be the significant fact in this connection. After destruction of the rapid mechanism, in the sole-plates or elsewhere, the mechanism of muscular contraction can only be brought into play in the slow mode, and with a correspondingly slow chronaxie. Even this slow mechanism is not,

however, exempt from attack after denervation, for its chronaxie is a function of the degree of wasting of the muscle, rapid wasting being accompanied by a corresponding increase in chronaxie, and perhaps by increasing sluggishness of contraction. This lengthening of slow time-characteristics in conjunction with atrophy raises the question, by analogy with nerve, of a possible inverse relationship of fiber size with chronaxie and with speed of contraction, when by denervation all special excitatory mechanism is eliminated and the time-characteristics are those of the fiber itself.

The fibrillation of denervated muscle is obviously a problem in altered excitability and contractility, and should be explicable in terms of the basic physiological alterations just described, but first the unit in action must be established, and its rate. A rate of contraction between 2 and 10 times a second in the individual fiber does not suggest the slow mode of response. On the other hand, if the contraction involves only a small portion of the fiber, adjacent portions, or adjacent fibers might respond in such time-sequence as to give an impression of this rate, yet the local state of contraction be considerably more enduring. Or recovery after such a local contraction might be very much more rapid than after contraction of the entire fiber. Since fibrillation continues for a year or more after denervation, it seems improbable that it operates in the rapid mode. If it does not, then, operate in the slow mode, it must represent a new form of response, which is unlikely.

The outstanding problem presented by fibrillation is the mechanism of excitation of this spontaneous rhythmical contraction. The coincident development of fibrillation and of acetylcholine sensitization focuses attention on the possibility of acetylcholine excitation. Since Dale, Feldberg and Vogt (1936) obtained no demonstrable release of acetylcholine on direct stimulation of denervated muscle, a source of acetylcholine adequate to maintain the muscle in continuous fibrillation for months presents a difficulty. On the other hand Bender and Kennard (1938) appear to have demonstrated that under exceptional circumstances of fright and sudden effort an acetylcholine-like substance circulates in the blood in concentration sufficient to excite the contracture mechanism of denervated facial muscle. If fibrillation represents a lesser degree of excitation of the same mechanism, the smaller concentration of acetylcholine necessary for this might possibly be available continually in the general circulation, if not locally. Denny-Brown and Pennybacker (1938) argue strongly in favor of the acetylcholine stimulus, supporting their argument with an observation credited

to Langley and Kato that physostigmine markedly enhances fibrillation in denervated muscle. Langley and Kato (1915a) themselves state, however: "In the considerable number of observations we have made on this point, we did not find any striking increase in fibrillation on giving physostigmine. Some effect appeared in nearly all cases to be produced but in view of the difficulty of observation, we feel no certainty that the appearance was not illusory." Transitory eserine augmentation was observed by Rosenblueth and Luco (1937), who nevertheless consider acetylcholine stimulation improbable.

Certainly an explanation for this steadily continued, rhythmical contraction which does not go outside the muscle would be more acceptable. In a relevant study of rhythmic discharges in skeletal muscle fibers Adrian and Gelfan (1933) have shown that local depolarization of muscle fibers tends to produce rhythmic contraction, although a slowed rate of adaptation is probably the significant factor. With the evidence at hand it is not possible to fix upon any one condition in denervated muscle which might underlie local depolarization. The largely increased calcium content might be suspect because Watchorn and Murray (1938) showed for the embryo chick's heart that a relative increase of calcium at the surface of the cell over potassium in the interior, if great enough, stops beating and induces fibrillation. Against this, however, as the mechanism of denervation fibrillation is the fact that Langley (1916) was able to stop the fibrillation with local or intravenous calcium lactate. Some far reaching surface change is, however, suggested by a variety of considerations. The susceptibility of fibrillation to anoxemia (Langley and Kato, 1915a) suggests that a restorative process, possibly at a surface, is far from vigorous at best, and easily becomes ineffective resulting in persisting depolarization and inexcitability. Likewise the increased sensitivity of the slow mode of response in denervated muscle to mechanical and electrical stimulation and to potassium chloride (Brown, 1937), that is, to stimulation in which acetylcholine sensitization has no suspected part, demonstrating as it does a basic condition of hyperexcitability which might very well give rise to spontaneous rhythmical contraction, can be best interpreted as a surface phenomenon, although what surface is involved is not indicated. The loss of nuclear orientation to the sarcolemma may be hesitantly recalled in this connection. Furthermore, if Denny-Brown's reported early changes in contractility are in any way analogous to the phenomenon behind chronaxie of subordination, they may similarly represent an altered state of polarization of the cell surface related very immediately to loss of nerve function.

Cannon and Rosenblueth (1936) have argued pertinently in this connection, that the general phenomenon of sensitization to neurohumors, specifically including acetylcholine sensitization of skeletal muscle, is only one example of the increased permeability of denervated structures to numerous stimulating agents; that is, one example of a surface alteration. Until all the possibilities intrinsic in the muscle have been investigated, it seems premature to fasten upon acetylcholinestimulation without unequivocal evidence of its presence.

Irreversible contracture may perhaps be considered the end-point of fibrillation. If fibrillation results from a state of partial depolarization of the muscle fiber however set up, and if this depolarization becomes more complete as atrophy progresses, then at the point at which the depolarization remains complete, contracture should develop. In this state the muscle fibers should be inexcitable,—an interpretation which agrees with clinical experience in man.

The neurohumeral mechanism of muscle is affected by denervation in two ways. Sensitization to acetylcholine might perhaps be easily accounted for if a choline esterase is concentrated in the neighborhood of the nerve endings of skeletal muscle, as Marnay and Nachmansohn (1938) believe, and disappears in large part as these degenerate, as Martini and Torda (1937) have claimed. The rate of disappearance of choline esterase given by Martini and Torda (1937) is too slow to correlate with destruction of the nervous tissue, but correlates well with the onset and course of destruction of the sole-plates. The difficulty with this interpretation is that Marnay and Nachmansohn (1937) not only failed to substantiate Martini and Torda, but found the choline esterase of muscle actually increased after denervation. However, if acetylcholine sensitization is only one example of a general condition of sensitization, as Cannon and Rosenblueth believe, including sensitization to such different substances as potassium chloride and nicotine, this eserine argument loses all point. Dale, Feldberg and Vogt's (1936) failure to obtain acetylcholine on direct stimulation of denervated muscle could result either from elimination of the acetylcholine producing mechanism following denervation,—whether correlated with nerve or sole-plate destruction their data do not indicate. Or it could result from continuous exhaustion of this mechanism by the continuous fibrillation, analogous with their failure to obtain acetylcholine from fatigued muscle. Considering the almost certain association of acetylcholine production with some portion of the nerve ending, nervous or non-nervous, this latter is very improbable.

The opening of the second phase of the reaction of muscle to denervation by the coincident onset of fibrillation and of atrophy at first confuses the analysis of mechanism, for either one may reasonably be postulated to underlie the other. If, however, fibrillation be considered to climax a progression of change in excitability and contractility perhaps beginning even at the moment of denervation, then the fibrillation takes precedence as Langley was inclined to believe. And the slight evidence of Fenn's figures on potassium, and Cathcart, Henderson and Paton's on creatine support this view. For the high values for these structural materials in the first phase, and their rapid depletion in the second, suggest that with the onset of fibrillation, the muscle is precipitated into atrophy from a previous state of rest and accumulation. The precipitate glycogen depletion described by Knowlton and Hines is in agreement with this, but because glycogen is less an essential structural material than a material stored for fairly immediate use, its depletion is less a demonstration of atrophy than of excessive use. The extraordinary depletion of phosphocreatine with a normal creatine content is probably the best gauge of the intensity of the fibrillary activity, representing a degree of fatigue which cannot be attained in the intact mammal. In this connection Fenn (1938) found that the depleting effect of electrically excited activity on the potassium of normal muscle was at a maximum with rhythmical contraction at a rate of 8 per second,—within the range of the rate of fibrillation.

Granting the precedence of fibrillation and its extraordinary intensity, atrophy is easily accounted for as driving of the muscle, inadequately protected by its refractory property, even to the point of utilization of its own structural materials. Less grave atrophy has, in fact, been described by Lorenz (1904) as developing in normally innervated, but overworked human muscle in relation to occupation. In late stages of denervation, the onset of contracture should terminate the exhausting demand, and perhaps help to preserve muscle tissue for the long periods for which it is known to survive in man. And if the contracture is only comparatively irreversible, like the contracture of local tetanus (Ranson and Ranson, 1929), permitting the muscle fibers to make a spontaneous recovery during the rest interval of contracture, individual fibers may, perhaps, alternate between contracture and inexcitability, and renewed excitability and fibrillation, although the muscle as a whole remains set in contracture. Such a condition would allow the survival for many years of a low-grade excitability and contractility of the muscle as a

whole, and the continuance of fibrillation suspected in Proebster's case, phenomena which are otherwise explicable only by assuming extraordinary differences between individual muscle fibers, differences causing some fibers to go into contracture two months after denervation, and others not after twenty years.

The middle phase of the reaction of muscle to denervation presents one last problem of mechanism in relation to the acute degenerative processes. The interjection into the orderly sequence of atrophy and fibrous metaplasia, of these processes, deferred and erratic as they are, clearly involves more complex conditions than uniform denervation. Their predilection for the central fibrils of deep lying fibers suggests a factor of poor nutrition or impaired elimination in their production, while their predilection for fibers adjacent to sites of intercurrent infection such as ulcers or trichina, suggests an exogenous toxic factor. These are compatible. The increasing incidence of these degenerations up to six months after denervation is easily credited, in a muscle working to exhaustion, to increasing interference with both nutrition and elimination by the fibrosis around capillaries and muscle fibers. Their subsidence after six months, in spite of the increasing fibrosis, is also explicable because as the muscle passes gradually into contracture, the nutritional demands on the tissue should diminish greatly, perhaps to the point where the supply is adequate, since almost certainly contracture involves no metabolism other than the maintenance metabolism of the tissue.

Within the muscle spindle, the atrophy and degeneration following motor denervation need no special consideration. The same factors may safely be assumed to operate intrafusally or extrafusally to the same ends. The changes following sensory denervation, on the other hand, present novel aspects. For if the sensory innervation is epilemmal as is usually believed, what influence is brought to bear by its destruction across the cell surface to destroy the equatorial nuclei? I, myself, (1932b) have questioned this separation of nerve and nuclei. The simplest explanation of the nuclear degeneration is probably to be found in the concept of Pallot (1934), that the equatorial development of nuclei and nerve ramifications is, like the motor end-plate, constituted of nerve and neurilemma tissues, the latter contributing the nuclei and granular cytoplasm. This ending is, he believes, applied around a very thin strand of myofibrillae. Degeneration of these nuclei would then be analogous to the degeneration of the central nuclei of the sole-plate,

and be a problem, not of nerve-muscle, but of nerve-neurilemma relationship. However, this interpretation of these nuclei is not generally recognized, not even by Couteaux (1938b).

The manifold aspects of the reaction of muscle to denervation now present themselves in simpler form. If atrophy and fibrous metaplasia on the one hand, and the acute degenerative processes on the other, all result from exhaustion of the muscle by the ceaseless fibrillation; and if fibrillation, and contracture, are the culmination of earlier changes in excitability and in contractility of the denervated muscle;—granted these changes, and in addition the factor of flaccidity for its effect on nuclear form, and the factor of time; then the reaction of muscle to denervation is fairly adequately accounted for. The question may now be considered: does this reaction demonstrate the preëxistence of a trophic control of skeletal muscle by the nervous system as has so generally been believed; and if so, what is its character?

Trophic control is, in the meaning of the words, nutritional control. Clearly, denervating skeletal muscle produces a nutritional disorder by imposing a demand in excess of the resources of the tissue. In contrast with cardiac muscle which may, apparently, fibrillate over long periods of time with impunity, skeletal muscle in fibrillation rapidly depletes its stores of glycogen and phosphocreatine and steadily reduces its mass until the exhausting demands are, perhaps, arrested by the onset of contracture. The question might be raised: why, with this unusual demand, the muscle does not hypertrophy. But the demand may be too great, or too continuous, or without proper sequence of tension and rest. Normal tension is, in fact, not created, while abnormal stretching must be constantly taking place. Langley (1916) first clearly recognized the analogy between denervated and fatigued muscle, but long before that the resemblance between the reaction of muscle to denervation and its reaction in extreme starvation had been repeatedly pointed out. In the presence of the fibrillation of denervated muscle, the concept of disuse atrophy becomes quite inapplicable even though it can be shown that muscle disused in other circumstances does atrophy. The same holds, likewise, for Bradley's (1938) concept of autolytic atrophy. Activity as such, though unquestionably a neurotrophic agent influencing both the development and maintenance of skeletal muscle, is not the neurotrophic agent eliminated by denervation.

Since fibrillation creates the excessive demand on denervated muscle, the nature of the normal control which is disordered by denervation should be evident in restraint of fibrillation. If it should be shown in

warm-blooded embryos that an original, spontaneous, rhythmical contraction is suppressed in conjunction with innervation of the skeletal muscle fibers, the suspicion that nervous innervation serves to restrain spontaneous activity throughout the life of the fibers would be reinforced. But this has not been done. Since the onset of spontaneous rhythmical contraction after denervation of fully developed muscle appears to climax earlier changes in excitability and contractility, the nature of the abolished control may more logically be sought in these. If it shall be proven that acetylcholine sensitization is the cause of these early changes, and if disappearance of choline esterase shall be shown to be the cause of acetylcholine sensitization, then choline esterase will qualify as a specific agent restraining spontaneous action; but the difficulties in the way of accepting this hypothesis have already been brought out. Moreover, little is gained by assuming acetylcholine stimulation of fibrillation alone, if the earlier changes require separate explanation. On the other hand, it does not seem possible to account for these early changes, and perhaps the resignation of sarcolemmal orientation by the nuclei, other than by postulating some specific agency in the motor innervation of skeletal muscle. This may involve substantial transfer from the nerve to the muscle, or it may employ other means of interaction. Certainly it is not activity as such. The agency exists as a conditioning influence which is essential to the maintenance of the normal physiological properties of the muscle fiber, notably the dominance of the rapid mode of response over the slow and the suppression of spontaneous contraction. Not improbably it determined the development of these in the embryo. Unquestionably this potent influence of the nervous system constitutes a proper neurotrophic control of muscle such that the motor innervation of skeletal muscle may be considered a trophic innervation. Nevertheless, it should be clearly recognized that this influence is only a part, although in warm-blooded animals the major part, of the total neurotrophic control of skeletal muscle, and that the use of the phrase describes a result produced, but is a very inadequate description of the means of its attainment.

On first consideration fibrillation presents itself as a unique condition incidental to the denervation of skeletal muscle, but this is not the case. The spontaneous fibrillation of denervated muscle can be matched with other instances of spontaneous activity of denervated tissues: with the paralytic secretion of glands, or with the reported spontaneous discharge of decentralized sympathetic ganglia (Govaerts, 1935, 1936) if this shall be confirmed. Moreover, although smooth muscle does not fibrillate

when denervated, the recovery of tone in denervated blood vessels, and possibly in the iris and nictitating membrane and other sites, may constitute a corresponding phenomenon of slower time characteristics. Furthermore, in all these cases the development of spontaneous activity is accompanied by sensitization to neurohumors and in all except the smooth muscle, by atrophy. If these analogies are correctly drawn, then the reaction of muscle to denervation is not a reaction peculiar to muscle, but a phenomenon of relationship between nerve and nonnervous tissues of broad significance.

REFERENCES

ADRIAN, E. D. The electrical reactions of muscles before and after nerve injury. Brain **39:** 1, 1916.

Physiological bases of electrical tests in peripheral nerve injury. Arch. Radiol. and Electrotherap. **21:** 379, 1917.

ADRIAN, E. D. AND S. GELFAN. Rhythmic activity in skeletal muscle fibers. J. Physiol. **78:** 271, 1933.

ADRIAN, E. D. AND D. R. OWEN. The electric response of denervated muscle. J. Physiol. **55:** 326, 1921.

ARLOING, S. ET E. CHANTRE. Effets de la section des nerfs du sphincter ani sur le rôle, les propriétés physiologiques et anatomiques de ce muscle et sur l'organisime en général. C. R. d. L'Acad. d. Sci. **127:** 700, 1898.

AUDOVA, A. Vergleichende Untersuchungen über die Chemischen Veränderungen bei der Muskelatrophie. Skand. Arch. f. Physiol. **44:** 1, 1923.

AVELLONE, L. Sulla funzione dei muscoli paralizzati mediante il taglio dei nervi motori. XIV. Variazioni del contenuto in fosforo, acido lattico, glicogeno. Riv. di Pat. Sperimentale **2:** 1, 1927.

AVELLONE, L. E G. DI MACCO. Sulla funzione dei muscoli immobilizzati mediante il taglio dei nervi motori. IV. Azoto totale, purine, aminoacidi, creatina. Ann. di Clin. Med. Sper. **15:** 39, 1925.

BARRON, D. H. Untersuchungen über die Chonaxie normaler und sympatikusloser Muskeln. Zschr. f. Biol. **95:** 567, 1934.

BATTEN, F. E. The muscle spindle under pathological conditions. Brain **20:** 138, 1897.

BENDER, M. B. Fright and drug contractions in denervated facial and ocular muscles of monkeys. Am. J. Physiol. **121:** 609, 1938.

BENDER, M. B. AND M. A. KENNARD. The fright reaction after section of the facial, trigeminal and cervical sympathetic nerves. J. Neurophysiol. **1:** 431, 1938.

BEZNÓK, A. B. L. AND Z. HASCH. The effect of sympathectomy on the fatty deposit in connective tissue. Quart. J. Exper. Physiol. **27:** 1, 1937.

BLOOR, W. R. Effect of activity on the phospholipid and cholesterol content of muscle. J. Biol. Chem. **119:** 451, 1937.

BOEKE, J. Studien zur Nervenregeneration. I. Verhandl. d. Konink. Akad. v. Wetensch. te Amsterdam, 2 sectie, D **18:** 1, 1916.

Bottazzi, P. Ueber die Wirkung des Veratrins und anderer Stoffe auf die quergestreifte, atriale und glatte Musculatur. Arch. f. Anat. u. Physiol. Physiol. Abth. 377, 1901.

Bradley, H. G. Autolysis and atrophy. Physiol. Rev. 18: 173, 1938.

Bremer, F. Researches on the contracture of skeletal muscle. J. Physiol. 76: 65, 1932.

Bremer, F. et P. Gérard. Conséquences de l'enervation des muscles striés dans la série des vertebres. C. R. Soc. d. Biol. 92: 1327, 1925.

Brissaud, E. Recherches anatomo-pathologiques et physiologiques sur la contracture permanente des hémiplégique. Thése pour le Doctorat en Médecine Faculté de Médecine de Paris, 1880.

Brown, G. L. The actions of acetylcholine on denervated mammalian and frog's muscle. J. Physiol. 89: 438, 1937.

The action of potassium chloride on mammalian muscle. J. Physiol. 91: 4p, 1937.

Buchthal, F., G. G. Knappeis und J. Lindhard. Die Struktur der quergestreiften, lebenden Muskelfaser des Frosches in Ruhe und während der Kontraktion. Skand. Arch. f. Physiol. 73: 163, 1936.

Cahn, T. Dégénérescence musculaire. Ann. d. Physiol. 3: 4, 1927.

Califano, L. Sulla funzione dei muscoli paralizzati mediante il taglio dei nervi motori. XV. Il consumo di ossigeno. Riv. di Pat. Sper. 2: 48, 1927.

Cannon, W. B. The autonomic nervous system. Lancet, May 24, 1109, 1930.

Cannon, W. B. and A. Rosenblueth. Sensitization of a sympathetic ganglion by preganglionic denervation. Am. J. Physiol. 116: 408, 1936.

Cathcart, E. P., P. S. Henderson and D. N. Paton. On the creatin content of skeletal muscle in degeneration following denervation. J. Physiol. 52: 70, 1918.

Cedrangolo, F. Azione del sistema nervoso sul metabolismo dei glicidi e dei lipidi nel muscolo. Arch. di Scienze Biol. 23: 377, 1937.

Chandelon, Th. Ueber die Einwirkung der Arterienunterbindung und der Nervendurchschneidung auf der Glycogengehalt der Muskeln. Pflüger's Arch. 13: 626, 1876.

Chen, K. K., W. Meek and H. C. Bradley. Studies of autolysis. XII. Experimental atrophy of muscle tissue. J. Biol. Chem. 61: 807, 1924.

Chor, H., R. E. Dolkart and H. A. Davenport. Chemical and histological changes in denervated skeletal muscle of the monkey and cat. Am. J. Physiol. 118: 1, 1937.

Coates, H. E. and O. W. Tiegs. Further observation on certain alleged effects of sympathetic nerves on skeletal muscle. Australian J. Exper. Biol. and Med. Sci. 7: 37, 1930.

Cook, D. D. and R. W. Gerard. The effect of stimulation on the degeneration of a severed peripheral nerve. Am. J. Physiol. 97: 413, 1931.

Couteaux, R. Sur l'origine de la sôle des plaques motrices. C. R. Soc. d. Biol. 127: 218, 1938.

La formation des plaques motrices et les correlation myoneurales au cours de l'histogènese musculaire. C. R. Soc. de Biol. 127: 571, 1938.

Dale, H. H., W. Feldberg and M. Vogt. Release of acetylcholine at voluntary motor nerve endings. J. Physiol. 86: 353, 1936.

DALE, H. H. AND J. H. GADDUM. Reactions of denervated voluntary muscle and their bearing on the mode of action of parasympathetic and related nerves. J. Physiol. **70**: 109, 1930.

DALE, H. H. AND H. S. GASSER. The pharmacology of denervated mammalian muscle. I. The nature of the substances producing contracture. J. Pharm. and Exper. Therap. **29**: 53, 1926.

DAVENPORT, H. K., S. W. RANSON AND E. STEVENS. Microscopic changes of muscle in myostatic contracture caused by tetanus toxin. Arch. Path. **7**: 978, 1929.

DENNY-BROWN, D. E. The histological features of striped muscle in relation to its functional activity. Proc. Roy. Soc. B **104**: 371, 1929.

DENNY-BROWN, D. E. AND J. B. PENNYBACKER. Fibrillation and fasciculation in voluntary muscle. Brain **61**: 311, 1938.

DUEL, A. B. Clinical experiences in the surgical treatment of facial palsy by autoplastic nerve grafts. Arch. Otolaryng. **16**: 767, 1932.

ERB, W. Zur Pathologie und pathologischen Anatomie peripherischen Paralysen. Deutsch. Arch. f. klin. Med. **4**: 535, 1868.

Zur Pathologie und pathologischen Anatomie peripherischen Paralysen. Deutsch. Arch. f. klin. Med. **5**: 42, 1869.

FELDBERG, W. Experiments on the degeneration of striated muscle fibers after sympathetic denervation. J. Physiol. **61**: xxxii, 1926.

FENN, W. O. The oxygen consumption of muscles made non-irritable by sugar solutions. Am. J. Physiol. **97**: 635, 1931.

Loss of potassium in voluntary contraction. Am. J. Physiol. **120**: 675, 1937.

Factors affecting the loss of potassium from stimulated muscles. Am. J. Physiol. **124**: 213, 1938.

FLORESCO, N. 1903. Influence de la résection du nerf sympathetique cervical sur les plaques motrices et les vaisseux des muscles. C. R. Soc. de Biol. **55**: 228, 1903.

FORSTER, L. Zur Kenntniss der Muskelspindeln. Arch. f. path. Anat. u. Physiol. **137**: 121, 1894.

GAISSINSKY, B. E. AND M. I. LEWANTOWSKY. Changes in striped muscle after sympathectomy. J. Physiol. **67**: iv, 1929.

GESSLER, H. Untersuchungen über die letzten Endigungen der motorischen Nerven im quergestreiften Muskel und ihre Verhalten nach der Durchschneidung der Nervenstamme. Deutsch. Arch. f. klin. Med. **33**: 42, 1883.

GOVAERTS, J. Apparition d'un tonus cardio-accelerateur dans le ganglion stellaire déconnecté centralement. C. R. Soc. d. Biol. **119**: 1181, 1935.

Etude oscillographique de l'activeté electrique du ganglion stellairé deconnecté du névraxe. C. R. Soc. d. Biol. **121**: 854, 1936.

GRUND, G. Zur chemischen Pathologie des Muskels. I. Stickstoff-und Phosphorverteilung im entarteten Muskel. Arch. f. Exper. Path. **67**: 393, 1912.

Über die Entstehung des fibrillaren Muskelzuckungen bei spinalen Amyotrophien. Deutsch, Ztsch. f. Nervenhk. **145**: 99, 1937.

HARRIS, K. E. AND G. P. WRIGHT. A case of haemangiectatic hypertrophy of a limb and observations upon rate of growth in presence of increased blood supply. Heart 15: 141, 1930.

HEIDENHAIN, R. Ueber pseudomotorische Nervenwirkungen. Arch. f. Physiol. Suppl. p. 133, 1883.

HINES, H. M. AND G. C. KNOWLTON. Changes in the skeletal muscle of the rat following denervation. Am. J. Physiol. 104: 379, 1933.

The influence of fasting and environmental temperature upon the atrophy of denervated skeletal muscle. Am. J. Physiol. 110: 8, 1934.

Effect of thyroparathyroidectomy and thyroxin on rate of atrophy of skeletal muscle. Proc. Soc. Exper. Biol. and Med. 31: 1029, 1934.

The rôle of the nervous system in the regulation of the glycogen metabolism of skeletal muscle. Am. J. Physiol. 111: 243, 1935.

Electrolyte and water changes in muscle during atrophy. Am. J. Physiol. 120: 719, 1937.

HINES, H. M., C. E. LEESE AND G. C. KNOWLTON. The metabolism of skeletal muscle undergoing atrophy of denervation. Am. J. Physiol. 98: 50, 1931.

HINSEY, J. C. Personal communication, 1936.

HOLOBUT, W. S. UND B. JALOWY. Die De- und Regeneration des peripheren motorischen Nervensystem auf einen morphologisch-funktionellen Grundlage. Zschr. f. Zellf. u. mikr. Anat. 25: 541, 1936.

HORSLEY, V. Short note on sense organs in muscle and on the preservation of muscle spindles in conditions of extreme muscular atrophy following section of the motor nerve. Brain 20: 375, 1897.

HUNTER, J. I. Lectures on the sympathetic innervation of striated muscle. Brit. Med. J. 1: 197, 251, 298, 350, 398, 1925.

HÜRTHLE, K. UND K. WACHHOLDER. Histologische Structur und optische Eigenschaften der Muskeln. Bethe's Handb. d. Normalen und Pathologischen Physiologie 8: i; 108, 1925.

JAMIN, F. Experimentelle Untersuchungen zur Lehre von der Atrophie gelähmter Muskeln. Fischer, Jena, 1904.

JONNESCO, T. ET M. FLORESCO. Phénomènes observés apres la resection du nerf sympathetique cervical chez l'homme. J. de Physiol. et de Path. gen. 4: 845, 1902.

KERPER, A. H. Histological changes in striated muscle following sympathetic denervation. Anat. Rec. 38: 18, 1928.

KNOLL, P. UND H. HAUER. Über das Verhalten der protoplamaarmen und protoplasmareichen, quergestreiften Muskelfasern unter pathologischen Verhältnissen. Sitzber. d. k. Akad. Wissensch. Wien. Math. Nat. Cl. iii 101: 315, 1892.

KNOWLTON, G. C. AND H. M. HINES. The respiratory metabolism of atrophic muscle. Am. J. Physiol. 109: 200, 1934.

Kinetics of muscle atrophy in different species. Proc. Soc. Exper. Biol. and Med. 35: 394, 1936.

Acetylcholine contracture of denervated muscle. Am. J. Physiol. 120: 757, 1937.

SHERRINGTON, C. S. On the anatomical constitution of nerves of skeletal muscle; with remarks on recurrent fibers in the ventral spinal nerve root. J. Physiol. **17**: 211, 1894.
Further note on the sensory nerves of muscles. Proc. Roy. Soc., B **61**: 247, 1897.
SPIEDEL, C. C. Studies of living muscle. I. Growth, injury and repair of striated muscle as revealed by prolonged observation of individual fibers in living frog tadpoles. Am. J. Anat. **62**: 179, 1938.
STEYRER, A. Ein Beitrag zur Chemie der entarteten Muskels. Beitr. z. Chem. Physiol. u. Path. **4**: 234, 1904.
STIER, S. Experimentelle Untersuchungen über das Verhalten der quergestreiften Muskeln nach Läsionen der Nervensystems. Arch. f. Psych. u. Nervenkrankh. **29**: 249, 1896.
TAKAHASHI, N. Untersuchungen über die tonisierenden und tropischen Funktionen des Sympathicus. Pflüger's Arch. **193**: 322, 1922.
TELLO, F. Dégénération et régénération des plaques motrices. Trab. d. Lab. d. Inv. Biol., Univ. Madrid **5**: 117, 1907.
La régénération dans les faisceaux de Kühne. Trab. d. Lab. d. Inv. Biol., Univ. Madrid **5**: 227, 1907.
TOWER, S. S. A search for trophic influence of posterior spinal roots on skeletal muscle, with a note on the nerve fibers found in the proximal stumps of the roots after excision of the root ganglia. Brain **54**: 99, 1931.
A search for trophic influence of the sympathetic nervous system on the adult mammalian skeletal muscle fiber. Johns Hopkins Hosp. Bull. **48**: 115, 1931.
The effects of sympathetic denervation of mammalian tissue during the period of postnatal growth. Am. J. Physiol. **100**: 295, 1932.
Atrophy and degeneration in the muscle spindle. Brain **55**: 77, 1932.
Atrophy and degeneration in skeletal muscle. Am. J. Anat. **56**: 1, 1935.
Unpublished observations on fibrillation in denervated skeletal muscle. 1938.
VAY, F. Ueber den Glykogengehalt der Muskeln nach Nervendurchschneidung. Arch. f. exper. Path. u. Pharmakol. **34**: 45, 1894.
WATCHORN, E. AND P. D. F. MURRAY. Loss of potassium from embryonic chick heart in potassium-free media with and without calcium. Proc. Roy. Soc., B **124**: 446, 1938.
WATTS, C. F. The effect of curari and denervation upon the electrical excitability of striated muscle. J. Physiol. **59**: 143, 1924.
WESTENBRINK, H. G. K. UND H. KRABBE. Über den Einfluss der Nervendurchschneidung auf die chemische Zusammensetzung der quergestreiften Muskels. Arch. Neérl. d. Physiol. **21**: 455, 1936.
WHIPPLE, G. H. The hemoglobin of striated muscle. II. Variations due to anaemia and paralysis. Am. J. Physiol. **76**: 708, 1926.
WILLARD, W. A. AND E. C. GRAU. Some histological changes in striate skeletal muscle following nerve sectioning. Anat. Rec. **27**: 192, 1924.
ZANGHI, G. Sulle variazioni del fosfogeno musgolare per effetto della stricninizzazione e della recesione dei nervi. Bol del. Soc. Ital. d. Biol. Sper. **3**: 723, 1928.

STUTTERING[1]

STANLEY COBB AND EDWIN M. COLE

Departments of Psychiatry and Neurology, Massachusetts General Hospital, Boston, Mass.

Man alone talks, and only in man is there a cerebral development sufficiently complex to serve as a mechanism for language. How this developed phylogenetically is admirably told by Elliott Smith in his *Essays on the Evolution of Man* (1). In phylogeny there is evident a close relationship between the development of binocular vision, manual skill, and finally a leading hand and leading cerebral hemisphere. When man's shrew-like ancestors took to trees and left behind them quadrupedal locomotion, their fore-limbs were emancipated to become hands, and the possibility of dexterity (with all that it implies) developed.

Other vertebrates specialize and reach narrow superiority by unique development of one type of sense organ. The hawk has a far better visual apparatus than man; the hound specializes on olfaction and lives in a world of smell; the bat has an auditory equipment beyond comparison. Man excels because of lack of specialization. His specialty is a leading hemisphere, evenly developed in the reception and elaboration of all types of sensory impression. It is this even development that allows for association between the different sensory receiving stations; and it is the development of the mechanism of association that has made intellectual behavior possible. Other vertebrates lost the possibility of intellect when they developed great excellence of one sense organ at the expense of an even development of all.

We have used the term "dexterity." Why is it "dexter," right? There is some evidence that rats are somewhat right handed (2) and crabs appear to tend that way, but in man alone is it definite. About seventy-five per cent of men are right handed, the remaining twenty-five per cent are left handed or ambidextrous (3). Various explanations have been given, from heliocentrism to blood-flow to the brain. None

[1] Stuttering and stammering are practically synonymous words; the former indicates the more repetitive vocalization, the latter the more blocked speech. The difference is unimportant.

are at all convincing. The most probable seems to be that it happens through the chance of Mendelian inheritance (4).

The dominance of the left hemisphere is marked in right handed people, but the functions are probably not confined to the left hemisphere; the dominance is relative. Recent studies (5, 6) have shown that even speech is subserved in a rudimentary way in the right hemisphere of right-handed persons. With a leading hemisphere in which there is a predominant centering of manual skill (praxia) symbolic understanding (gnosia), and language, there is great need of many associative tracts. The function of language needs both praxia and gnosia, in fact, each needs the other two for effective behavior. Nevertheless it can be taken as a proposition that language is the most highly integrated of man's functions. Although the integration is most complex at the cerebral level, it is integrated at several lower levels (7). Schematically one may divide these into five: the *neuromuscular* level takes in the peripheral muscles of speech in larynx, lips and tongue, with nerve cells in the medulla oblongata and axones to the muscles. Disturbances of this level lead to paralysis or partial paralysis of the speech mechanism with different degrees of aphonia. *Cortico-bulbar* neurones make up the next level and lesions of these (most commonly in the cerebral peduncles) cause dysarthria. The *cerebellum* is a coordinating mechanism of great importance in speech. Lesions here result in asynergia causing such symptoms as "scanning," explosive and monotonous speech. Only at the *cerebral* level do agnosia and aphasia result from lesion.

More complex than any of these fairly well understood mechanisms is the level of language that is disturbed in stuttering and stammering. Little is known of its cerebral localization. Evidence indicates that it is closely connected with emotional expression, and with dominance of one cerebral hemisphere, or lack of clearly defined dominance. The physiology of this fifth and highest level is unknown. At best, it is sketchily indicated by clinical data.

So much has been written about the causes and cure of stuttering that, in a short article of this sort, full justice to all points of view cannot be given. Nevertheless for the sake of simplification the principal schools of thought can be grouped into three categories and discussed in that way.

I. The older physicians dealt with stuttering as a disturbance of the peripheral speech mechanism (8). There is much to recommend this approach to the problem, for the presenting symptoms obviously con-

sist of disturbed functions of lips, jaws, tongue, throat and chest when the stammerer speaks. Physicians, elocutionists, and other teachers studied the movements requisite for proper sound production. Drills were devised to overcome the "improper" speech patterns which the stutterer had developed. It was thought that spasms of certain muscles (which are indeed present) caused the impairment of speech. A variety of exercises to promote muscular relaxation were used (9). Frequently physicians went so far as to perform operations to overcome stuttering. Even today in our clinic we see children who come in because of stammering who have had recommended or performed as therapeutic measures tonsillectomies, adenoidectomies and cutting of a supposed "tie" beneath the tongue. We have not seen relief of the symptoms as a result of such operations.

Speech teachers felt that stutterers had not mastered the movements required for making some sounds. These patterns were explained to the patient, and he was given drills in articulation. Others felt that speech would become normal if the difficult sounds were avoided. Consequently speech was practiced avoiding certain troublesome consonants or all initial consonants. An easy vowel sound was sometimes used as a start (10).

It was considered by some that the respiratory mechanism was at fault. These workers observed that when speaking the stutterer's respirations were irregular, or he talked during inspiration or at the end of expiration when there was insufficient air in the lungs to make proper sounds (11). Consequently breathing exercises, timing with a metronome, blowing balloons or candles while talking and other similar methods were used.

Certainly more therapists have attacked stammering from the point of view of faulty speech mechanics than from any other single angle; and probably more stutterers have been helped by these methods than any other. The results indicate that here is at least one vulnerable point of attack, and it should be respected and investigated. Unfortunately, many therapists are prejudiced and ride hobbies; they consider all methods except their own as "superficial." "The proof of the pudding is in the eating," however, and in the light of actual results, one must admit that a great many stammerers are helped by speech and breathing exercises, but we know of some that have been made worse. Whether the good results are due to the training or to the suggestion and psychological situation is a moot question.

II. From the mechanical school developed the psychological school.

Forerunners of this point of view were those who long ago considered stuttering a "habit": the result of a fright either pre- or post-natal, or of imitation of other stutterers. It was recognized that the speech mechanism might not be at fault as much as its control by the mind.[2] The development of this point of view has coincided with the awakening of scientific interest in psychological disorders and with increased insight into the fact that the "body" and "mind" are not separable, but one integrated unit.

From this newer approach, stuttering is considered as a disorder of the personality (12). As such the disturbed speech becomes a symptom of an underlying conflict, a maladjustment or abnormal emotional state, in short, a neurosis (13). It is said that the symptom is the result of fear or lack of confidence. Evidence for or against that theory is difficult to evaluate. Many of the authors merely express convictions and present little or no data. At present the best way to judge of the etiological theories is to examine the therapeutic results.

Sometimes the whole therapeutic effort is to restore confidence and banish fear of talking. Suggestion is knowingly employed. The stutterer is drilled to master a few sounds, words and then speeches; successes are emphasized and confidence is built up. It is probable that the drills and exercises do much more than just this; they give practice in well coördinated speech and put bad habits into the discard by forming new and better ones. A common trick that often works for a time is the association with speech of some habitual movement: the stutterer learns that a tap of the foot, nod of the head, whistle, or singsong inflection will get him started on a difficult word. Many stutterers develop such tricks for themselves and the reassurance and helpful suggestion of this "key" which unlocks speech, should not be minimized. It is impossible even to mention all the confidence-restoring methods employed. That they appeal to many is testified to by the large number of parents, teachers and physicians who try to encourage the stuttering child by telling him that he will soon "outgrow" his difficulty. It is easy for the physician to tell the patient to "forget it," but such reas-

[2] The term "functional" is commonly, but loosely applied by many physicians to mean "psychological." Scientific workers must be careful to speak accurately even at the risk of being pedantic. Accurately speaking *almost all neurological symptoms are "functional,"* because they are phenomena of a disordered function. To the physiologist this seems too elementary to be worth stating. Practically it is an axiom that must be reiterated to students because of the loose thinking of their clinical instructors.

surance usually results only in the patient's rightly losing confidence in the medical profession.

The psychological explanation of stammering is surely a complex affair that cannot be described simply as fear and lack of confidence. Attempts are being made to be more scientific, but many investigators seem to be willing to generalize on as yet inadequate data. It is claimed that stutterers have a "type of personality" which leads to their particular speech disturbance.[3] Others describe stuttering as an oral neurosis (14); still others emphasize the fear element or the motor "tic" phenomena. A comprehensive view is much needed

Adherents of the psychological viewpoint feel that much can be accomplished by reëducation of the patient along many lines. This is done by personal interviews, discussions of personal problems and a variety of speech activities. These restore confidence, give the patient a social rapport he has formerly lacked and in many ways satisfy cravings which have remained unsatisfied in the stutterer, perhaps because of his disability. Institutions have been established the purpose of which is to eradicate the patient's fear of speech and to care for his needs in respect to contact with his fellows (15). Along these lines we find many stutterers helped. One need only visit such a school for stammerers, and observe the enthusiastic support and perhaps desperate hope of some of the students, to realize the service it provides. A patient who went to one of these schools writes as follows:

> The program of the day consists in having three to four classes of about fifteen people. We read out loud very slowly, give short speeches in a slow drawl and have breathing exercises. We have a class of physical exercise to music, swinging our arms and legs. The atmosphere all the time is very happy and carefree, our handicaps forgotten. Speech is not emphasized at all and we are always told to forget it and that we don't want it. Speech comes after we have gotten control of our emotions, intellect and physical activity. These have to be balanced perfectly before speech comes. We have two evenings a week where we all gather in a hall and make speeches, tell funny stories or anything. I've spoken in this manner and have had hardly any difficulty.

Methods of treatment include conversations, speeches, dramatics, music, "rhythms," dancing and other activities which are designed to aid the patient in socialization and which afford considerable opportunity for exercising language. Psychological reëducation of the individual patient along many lines is the prime purpose. The enthusiasm and personality of the director is of great importance. The suc-

[3] Heredity plays a part in this, and is discussed below.

cesses are probably to be explained by the tolerant and eclectic approach, but the scientifically minded investigator can learn little about etiology from so complex a picture.

Other workers in the psychological fields, considering stuttering a neurotic symptom, have recommended a more special type of therapy. As an anxiety neurosis stuttering has been studied by the various schools of psychological analysis (16). Both psychoanalysis and less rigorous forms of individual therapy have been used. In all of these stuttering has been considered a symptom of an emotional disturbance amenable primarily to individual, psychological treatment consisting of repeated interviews with the patient in which he brings up his emotional difficulties and works them out with the aid of the analyst. The treatment may take five hours a week for six months or for two years. The Adlerian school of "individual psychologists" believe in studying the relation of the patient to his environment especially to his parents, siblings, work and ambition (17). This analysis of the conscious situation, with some dipping into the less conscious reactions as brought out by dreams, is often helpful in a few months. The Adlerians consider that the main causes of neurotic symptoms are an "inferiority complex," a "will to power" and an abnormal striving for superiority because of feelings of insecurity rooted in childhood experience. There appears to be much truth in this simple formulation especially when viewed in the perspective of our present competitive civilization. The type of analysis given by these individual psychologists tends to lessen the patient's feeling of tension; he feels less hurried, gains stability and poise by meeting some immediate problems, and often (much to his surprise) stops stammering. During treatment mention is rarely made of the speech defect; it is looked upon merely as a symptom that will disappear when emotional disturbances are alleviated.

Blanton (18) gives the best description of the psychoanalytic approach to stammering. With his wife as a colleague he has worked for years at various aspects of speech disorder and has formulated his results in a book *For Stutterers* (19). They believe that stuttering is merely a symptom, the expression of an abnormal emotional state, an anxiety resulting from inadequate emotional adjustment to the childhood environment. In each stutterer there is a particular cause for the neurosis which expresses itself overtly in the "stutter." Treatment of stuttering is, therefore, directed primarily at the cause of the neurosis. The essential factors in treatment are readjustment of environment,

individual guidance and psychoanalysis. The age of the patient and a variety of circumstances determine where the main emphasis shall be placed.

The mechanistic theory upon which the Blantons base their treatment seems to be the following: the "parts of the body that are utilized by speech are only borrowed for speech and have their first and most vital use in some other field of activity," such as chewing, sucking, swallowing, vomiting, breathing, just as the primary use of the hand is for holding objects and is later used for writing after intricate training. The cerebral control of speech, at first quite conscious, becomes more automatic through the establishment of normal neuro-muscular patterns both in the cortex and in the thalamus, basal ganglia and brain stem. The physical symptom, stuttering, is attributed to "an emotion that blocks off" the "control of the cortex over the thalamus and lower nerve centers and allows the primitive patterns" (infantile oral activities) to assert themselves and supplant normal speech movements.

We consider this theory plausible enough, but there is no good evidence to support it. Neurologizing is a useful pastime if it leads to further research, but all too frequently such theoretical speculations are quoted and soon accepted as facts.

On the basis of their observations and theories the Blantons have developed a course of treatment. Special emphasis is placed on the prevention of emotional aberrations and anxiety states in early childhood, and on the early recognition and treatment of stuttering. In the child, treatment is a "community project, the community consisting of the person with the disability, the members of the family, the teachers, and someone in charge who understands the problems of adjustment." The approach to the problem should be entirely one of readjustment of the child to its parents, playmates, routine and its general environment. Retraining of speech should be omitted. In the adolescent and adult, treatment is more of an individual and personal matter, and here psychiatry and psychoanalysis play dominant rôles.

The time and therefore the money necessary to obtain a psychoanalysis eliminate this type of therapy as a generally practical solution of the stutterer's problem. As psychiatric out-patient clinics develop it is hoped that treatment of this sort can be offered to those without wealth.

The therapeutic approach to stammering, as illustrated by the mechanistic school (I) and the psychological school (II), seems largely to depend upon the therapist's concept of the cause of the disorder. Because of the vagueness of the etiological conceptions and because

human reactions are extremely complex, the forms of therapy overlap. The simplest speech drills cannot be used without the establishment of some sort of a rapport between therapist and patient which must have some bearing on the therapeutic results. Similarly, the psychoanalyst provides a daily hour of speech exercise under ideal conditions of relaxation.

III. There is a third point of view in regard to stuttering, which, recognizing other etiological factors offers a different therapeutic approach. Speech production is looked upon as but one part of a complex and phylogenetically new skill, i.e., *language*—including not only speaking, hearing and understanding speech, but also reading and writing. The close association of these processes is clearly demonstrated in studies of aphasia where the whole language function or any part of it, may be disturbed. It has been demonstrated that certain areas in the brain must be intact if the language function is to be normal. These vital areas exist to a great extent in one side of the brain; the opposite side, though apparently idéntical in structure, is not essential for the carrying on of the processes of language. The aphasias and allied disorders are due to demonstrable cerebral lesions which prove that language depends upon the structural physiological integrity of certain areas in one cerebral hemisphere. No lesions have been demonstrated in the brains of stutterers. Nevertheless when one investigates carefully the family histories, one is struck by the frequency with which language disturbances recur in families. They include motor speech delay, stuttering, reading disabilities and possibly cases of auditory confusion in which the understanding of speech is impaired without deafness. The evidence is impressive (20) that many of these conditions are familial, which strongly suggests that many cases of stuttering are caused by some as yet unrecognized structural defects of the brain, because only structure is inheritable. If neurosis is defined as maladjustment, i.e., environmental, then neurosis cannot be the sole cause of stuttering.

Associated with stuttering and the above-mentioned speech defects of unknown etiology there is a familial tendency to left handedness. The hemisphere dominant in respect to handedness is usually dominant in language. The result seems to be that a defect in dominance is often associated both with ambidexterity, motor awkwardnesses and language disturbances. When the dominance is distinctly right-brained strong left handedness results, but this is less likely to be associated with speech defects than the states of mixed dominance. It seems probable that the

person whose inherited cerebral dominance is not clearly defined has a fusion of the impulses from the two hemispheres and thus has a relatively inadequate equipment for the perfection of linguistic skills. From this standpoint the stutterer is seen as a person constitutionally vulnerable in language. He may stutter, his brother or uncle or cousin may read and spell poorly, while his other brother may not talk until he is three or four years of age. All are manifestations of the same defective inherited equipment.

This conception of the cause of stuttering suggests a special therapy (21). A careful history will reveal the hereditary factors which are felt to be relevant and may also show that the stutterer was trained away from his master hand. This used to be the practice when left-handed children went to school, but it still is often met with in the form of "helping" a child to use his right hand, if he seems only a little lefthanded or perhaps ambidextrous. A thorough examination of the patient's use of his hands, feet and eyes will often show which really is his dominant hemisphere. Generally in a young child who is known to be a converted sinistral—(taught to use his right instead of his chosen left hand)—stuttering can be relieved by retraining in the use of the left hand. Many stutterers, however, give no such history of conversion and may, in fact, be apparently purely right sided in their dominance. Here, though the family history will give evidence of mixed cerebral dominance, no change of handedness is to be thought of. Other means of reënforcing the inadequately established cerebral dominance are resorted to. This is done by working through allied processes, which in the case of one language function, would be the other language functions. Help for the stutterer, then, comes from phonetic drills, both reading and sounding, from oral reading and from making letters with a pencil while reading or speaking. In this way speech, which is faulty, is reënforced by reading and writing and speech drills. The work done by Orton (22, 23) has pointed the way in this type of therapy.

In summarizing the problem of the etiology and treatment of stuttering one comes to the almost universal formulation of human biological problems—that of "the seed and the soil." That the inheritance (seed) is faulty in many cases of stuttering there can be no doubt. Therefore there is presumably a structural abnormality[4] in the brain. Persons with such abnormality are less adept in acquiring language and

[4] The word "abnormality" is used here advisedly and in its true sense of "variation from the norm." We do not consider that these common variants of *dominance deserve the term "defect."*

other motor skills. If the environment (soil) in which such a person is nurtured is difficult, especially if emotional stress is imposed on him in childhood, he is much more likely than his structurally normal neighbor to develop speech defect. It must be remembered that language is the most highly integrated function of the brain, and as such is the most sensitive indicator of emotions and the most vulnerable.

There are no satisfactory data to show how many cases of stuttering occur in children with normal cerebral dominance, and how many in those with mixed dominance. The tests are not clear cut and the limits of "normal" have not been drawn. We have no doubt that stuttering occurs in children born with normal brains, but we are convinced that those with mixed dominance are common, and are more likely to stutter or stammer if put under the sort of environmental pressure that causes neurosis (24). A case in which the boy escaped such pressure and never became a stutterer is briefly described in the autobiographical note of a well known professor of psychology:

> My father certainly had a conflict of dominance, showing itself in manual and visual habits, though further details are not now accessible. My mother seems to have been without such characteristics. I suspect that they exist to some extent in my only (younger) brother but they are not obvious. I recall in my father a very slight stammer, in my brother a distinct one when he was a child, but not as an adult. He had much difficulty in learning to read at school, but home training overcame it. He teaches English now. I seem to have been naturally left-sided; today I always use the left leg most in climbing, keeping most articles in left side pockets, steer a car entirely with the left hand. I naturally throw a ball with the left hand, learned to throw about equally with the right. I learned to write with my right hand, and recall no difficulty. I used kinasthetic cues, writing "in the air," to the amusement of my elders. Also I must have learned to read early and easily, and have always been a rapid, though rather unretentive reader. It has always been difficult for me to distinguish right and left orientations. As a child I learned to tell my right hand by a small mole that was on it; and now my memory image is of that mole on my left hand. I tended to reverse *letters*, like G and S; and in (Pitman) shorthand which I took up at about 38, had great difficulty in right and left orientation of strokes. Once I mentioned this to the instructor and was surprised that my difficulty seemed an unusual one. In school military drills I was especially prone to turn to the wrong side. I believe there is also a tendency to confuse even the words right and left in giving directions. Thus at motor levels I seem to present a rather marked instance of conflicting dominance.
>
> On the other hand, as above, I had no difficulty in learning to read. I stammered somewhat at about 13–14 years, though only traces remain of it now, mostly under emotional stress. From an Adlerian standpoint it may be of interest that the topic of language had early attraction for me as a study. It was via philology that I came into psychology. Today some of my friends give me credit for con-

siderable facility in imitating foreign accents of English. Mechanical relationships have always been relatively difficult, and suggest that a *conflicting* dominance might be a factor in lack of mechanical aptitude vocationally. It must be emphasized as a *conflict* of dominance, owing to the widespread superstition about left-sidedness (our figurative "sinister"; "left-handed compliment"). Actually some of the best personalities I have known have been pronouncedly left-handed.

In my own case the conflict of dominance has shown but sporadic effects on the language mechanism, manifesting itself mainly and markedly in more massive motor functions of a unilateral character. While I would claim no unusual freedom from conflict at higher levels, I know of no reason to attribute them to factors of bilateral dominance.

From the therapeutic standpoint it is obvious that the first need is for more accurate diagnosis. When one finds obvious ambidexterity or other symptoms of mixed cerebral dominance in a child with speech difficulty, the procedures devised by Orton should be used. Usually, however, the neurological indications are not clear cut; one suspects an abnormality of dominance but cannot prove it. The more refined observations of Orton (25) and Travis (26) have not yet been corroborated nor proven to be practical therapeutic guides. For young children without obvious neurological evidence of mixed dominance, the best therapy is to treat the parents. Make them see the necessity of a quiet environment with an atmosphere of security for the child. Pay no attention to the symptoms, but arrange for reading and singing in unison, which the child will enjoy and from which he will derive confidence in speaking.. Add to this careful general hygiene and a minimum of competition at home and at school. Such a regime if really carried out causes the symptoms to disappear in most cases.

Simple as this regime sounds, it is often impossible to obtain. The home situation may have elements of insecurity and tension that are incurable; the school may be hopelessly competitive. If the child cannot be sent away, the only thing to do then is to wait; when the stutter becomes a real impediment and the child grows old enough to want help, he can be treated individually. Then intelligently devised speech drills will help to combat bad habits of breathing and phonation that have resulted from stuttering; they will also build confidence. Any method of overcoming the block is better than no method, but the wise therapist avoids the tricks, and adheres to physiological principles. To make speech an art, a new learned skill, with proper expression, intonation and resonance is the aim. Elocution as usually taught is useless and often harmful. Practicing a skill is the best way of perfecting it. But

there are some cases where this very practice seems to increase the introspection and self-consciousness; the anxiety and the neurotic element are predominant. Here the therapy must be adapted to the special case, and wise psychological treatment instituted. Often this is impossible in youth. Perhaps sending the boy away to one of the better "stammering schools" will help, but most of these ride one hobby too hard and fail to consider the patient as a whole human being.

In adults, when the symptom has persisted past the age of twenty, the problem may be less difficult than in adolescence. The wish to be cured has by that time usually been accentuated by economic necessity, for stuttering is a most incapacitating symptom in the modern world. At this age in some cases the sort of treatment described by Blanton (19) is the most hopeful. The psychoanalytic therapy should ease the emotional tension; some speech drill will cure bad phonetic habits, and group work will help in the needed socialization. The fact that psychoanalysis is recommended for adults who stutter does not mean that we believe that stuttering is a neurosis. It merely means that in most adults there is little to be done about the inherited factor and the neurosis has become the greater problem. Some adults, however, respond well to the type of treatment referred to as neurological, based on the theory of cerebral dominance.

SUMMARY

Language is one of the most highly integrated functions of man, hence it is one of the most vulnerable functions and speech disorders are common. Stuttering becomes an impediment of greater or less degree in about one per cent of the adult population; many more pass through a period of stuttering in youth.

The neurological mechanism that causes stuttering is not known, but there is good evidence that a defective cerebral equipment is present in many stutterers. The most impressive evidence is that of heredity, for the symptom runs in families, and, if inherited, must have a structural neural basis. The relationship of this and other types of speech defect to ambidexterity has been demonstrated. All appear to be due to some variation from the normal in cerebral structure, but no anatomical abnormalities have yet been found.

Because language is a social function, and because speech is so closely related to emotional life, stuttering has important psychoneurotic aspects.

There is good evidence that emotional tension and stress in youth

precipitate stuttering, and that relief of such tension relieves the symptom. This does not prove, however, that the symptom is "purely neurotic," although treating the neurosis may be the most effective method of removing the symptom. Three principal types of therapy are employed, with many variants and amalgamations: *a*, the mechanistic school, which relies on speech exercises, but unwittingly uses a good deal of suggestion; *b*, the psychological schools, where repeated interviews are used to ease emotional tensions, but where incidentally much speech exercise comes in; and *c*, the neurological school where exercises aimed at correction of weaknesses of cerebral dominance are employed. Obviously the best therapy is to be found when the physician is eclectic; when, knowing all the methods and his patient, he applies an individual treatment.

REFERENCES

(1) Smith, E. G. Essays on the evolution of man. Oxford University Press, London, 1924.
(2) Tsai, L. S. and S. Maurer. Science 72: 436, 1930.
(3) Wile, I. S. Handedness: right and left. Lothrop, Lee and Shepard Co., Boston, 1934, 439 p.
(4) Jordan, H. E. J. Genetics 4: 77, 1914.
(5) Nielsen, J. M. Agnosia, apraxia, and aphasia. Los Angeles Neurological Society, Los Angeles, 1936, 210 p.
(6) Von Hagen, K. O. Bull. Los Angeles Neurol. Soc. 3: 98, 1938.
(7) Cobb, S. A preface to nervous disease. William Wood & Co., Baltimore 1936. *See figures 7 and 12.*
(8) Scripture, M. K. Quart. J. Speech Education 8: 145, 1922.
(9) Patzay-Lieberman, L. Monatschr. f. Ohrenh. 71: 1188, 1937.
(10) Robbins, S. D. Stammering and its treatment. Boston Stammerers' Institute, Boston, 1926, 121 p.
(11) Robbins, S. D. New England J. Med. 205: 146, 1931.
(12) Greene, J. S. N. Y. State J. Med. 36: 757, 1936.
(13) Fenichel, O. Outline of clinical psychoanalysis. W. W. Norton & Co., New York 1934, 491 p. Chapter VI.
(14) Coriat, I. H. Stammering, a psychoanalytic interpretation. Nervous and Mental Disease Monograph Series, no. 47, New York 1928, 68 p.
(15) Greene, J. S. J. A. M. A. 104: 2239, 1935.
(16) Appelt, A. The real cause of stammering and its permanent cure. Methuen & Co., London, 1911.
(17) Wexberg, E. Handbuch der Individualpsychologie. Bergmann, München 1926. Vol. I. Individual psychology, Cosmopolitan Book Corp., New York, 1929.
(18) Blanton, S. Mental Hygiene 15: 271, 1931.
(19) Blanton, S. and M. G. Blanton. For stutterers. D. Appleton-Century Co., New York 1936, 191 p.

hensive consideration of the vast literature that has arisen about this subject since the experiments of Heidenhain (1874) suggested that it might be important in the formation of urine. Nor will we adhere strictly to the chronological sequence of the work that has contributed to our present knowledge of this phase of renal physiology.

Renal tubular excretion is in general characterized by the circumstance that a preformed, diffusible solute is transferred across the tubule cell under such conditions that there results an increase in the free energy of the system in the form of a concentration gradient. The energy for such processes arises locally, being derived from the metabolic activity of the cells. The aspects of this subject to be considered are:

1. The general methods of approach to the problem with special reference to those which have resulted in the acceptance of tubular excretion as necessary to explain certain phenomena observed in the formation of urine.

2. A consideration of the nature of these processes; that is, their mechanism and their cellular limitations.

3. Their physiological importance in the economy of the organism.

4. The uses that they have been, or may be put to, in the study of the physiology of the kidney.

METHODS USED IN THE STUDY OF TUBULAR EXCRETION. Perhaps the simplest technique that sought to answer the question of whether tubular excretion was a factor in the formation of urine was the direct microscopical examination of kidneys during the elimination of colored or fluorescent substances. These experiments were largely confined to the kidneys of the fishes and the amphibia which, because of their loose anatomical structure, are ideal material for this type of examination. Except for certain special preparations to be mentioned later, the criteria used to evaluate the presence or absence of active tubular transfer are inadequate for this purpose (q.v. 48). Without knowledge of the extent of water reabsorption it is impossible to be certain in any particular case that a high concentration of dye in the tubular lumen is due to a process of tubular excretion; it might as well be caused by the concentration of glomerular filtrate by the tubular reabsorption of water. The demonstration of a high degree of cellular staining is likewise uncertain evidence of tubular excretion. Localization in the cell cannot in itself be taken as evidence that this process is one of storage which precedes or accompanies excretion into the lumen. Since entrance into the cell may be from its luminal border instead of from the interstitial fluid, this possibility must always be considered when inter-

preting simple cellular localization (36, 38). This mode of entry has been denied for certain substances, as for example indigo carmine (39 and others), because of the inability of the observer to visualize it in Bowman's capsule. The weakness of this evidence is apparent from the subsequent demonstration of the glomerular elimination of this substance in the frog by direct capsular puncture (70). It was later pointed out that, while there was some tubular excretion of indigo carmine, Heidenhain's error regarding the glomerular elimination of the dye followed from his not considering its tinctorial properties and the possibility of plasma binding (46). Furthermore, it is now known by direct evidence that cellular accumulation in the case of some dyes is due to the simple process of vital staining and in some others to a type of seemingly specific accumulation that does not necessitate concomitant or subsequent extrusion into the lumen (13).

A more critical use of direct observation combined with a more careful control of experimental variables characterized the work of Richards and Barnwell (1927). They demonstrated directly that frog's renal tubule is capable of taking phenol red into its lumen through its wall under conditions that exclude the participation of glomerular function, and that KCN is capable of inhibiting this activity. In these experiments glomerular function was eliminated in the nephrons under examination by perfusion of the tubules from below, pressure on the glomeruli, excision of the heart or of the kidney itself. Phenol red was applied directly to the surface of the tubule mass or indirectly through renal portal perfusion at low pressure. Given adequate oxygenation, the tubules of all preparations collected phenol red in their lumina, and in most cases the phenol red was in high concentration. It was pointed out, however, that tubular excretion might not be the necessary mechanism producing these results. They believed that they were as easily explicable on the basis of inward diffusion of water and dye or dye alone at one level of the tubule with subsequent concentration by the reabsorption of water and retention of dye at a lower level. In this view the active function of the tubule is not related to the entrance of the dye but to its retention and the active extrusion of water. They further pointed out that in the kidney of the living animal, *in situ*, it is inconceivable that the concentration of the dye can be increased directly by inward diffusion, since, by reason of glomerular elimination, the tubular fluid already contains it in concentration as high as, or higher than, that in which it exists in plasma water. (It may be noted that this suggestion as to the mechanism of transfer of phenol red must be

the aorta does not exclude the glomerular capillaries from the circulation under the conditions of Nussbaum's experiment (45). It was shown that they receive a significant collateral blood supply from one of the two divisions of the normal arterial supply to the ureters. The administration of urea to a Nussbaum preparation results in the reestablishment of glomerular circulation through the dilatation of these collateral branches. The ligation of these, as well as all other arterial branches to the kidney, produces a preparation that maintains its anuric state following otherwise effective doses of urea. It had been earlier shown that the single or double perfusion techniques described above do not effect a clean separation of the fluid introduced into the arterial and renal portal blood supplies of the kidney (69). This is in keeping with the known pressure relationships of the glomerular circulation and the renal portal system (37). The fundamental premise underlying all attempts at the establishment of tubular excretion by these techniques, that substances introduced into the renal portal system are not available for glomerular elimination, is therefore untenable.

In certain species of amphibia and reptiles methods have been developed by Richards and his co-workers which permit the study of renal function by direct observations on the resultant of activity of various parts of the nephron. The early work (67) was concerned with the nature of glomerular elimination, the glomerular fluid being collected quantitatively by capsular puncture. By the adaptation of chemical methods to the determination of the concentration of solutes in minute volumes of solution, it was possible to determine the concentration of certain constituents of glomerular filtrate and hence the rate of excretion of a substance by a single glomerulus. The demonstrated equality of all diffusible constituents in plasma water and glomerular filtrate, except for the small Donnan effect, is directly opposed to the historically important experiments of Heidenhain (1874) mentioned previously. It removes the necessity of accepting that the elimination of indigo carmine in the frog is by a purely tubular process (70). However the demonstration of this equal distribution in the case of urea did not warrant the conclusion that tubular excretion is not necessary to account for its renal elimination in the frog (104) as had been previously suggested (54). The further elaboration of these procedures permitted, by means of tubular puncture, a study of the concentration relationships in the remaining portions of the nephron (71). It was shown in this and other types of experiments that urea is concentrated more than

preting simple cellular localization (36, 38). This mode of entry has been denied for certain substances, as for example indigo carmine (39 and others), because of the inability of the observer to visualize it in Bowman's capsule. The weakness of this evidence is apparent from the subsequent demonstration of the glomerular elimination of this substance in the frog by direct capsular puncture (70). It was later pointed out that, while there was some tubular excretion of indigo carmine, Heidenhain's error regarding the glomerular elimination of the dye followed from his not considering its tinctorial properties and the possibility of plasma binding (46). Furthermore, it is now known by direct evidence that cellular accumulation in the case of some dyes is due to the simple process of vital staining and in some others to a type of seemingly specific accumulation that does not necessitate concomitant or subsequent extrusion into the lumen (13).

A more critical use of direct observation combined with a more careful control of experimental variables characterized the work of Richards and Barnwell (1927). They demonstrated directly that frog's renal tubule is capable of taking phenol red into its lumen through its wall under conditions that exclude the participation of glomerular function, and that KCN is capable of inhibiting this activity. In these experiments glomerular function was eliminated in the nephrons under examination by perfusion of the tubules from below, pressure on the glomeruli, excision of the heart or of the kidney itself. Phenol red was applied directly to the surface of the tubule mass or indirectly through renal portal perfusion at low pressure. Given adequate oxygenation, the tubules of all preparations collected phenol red in their lumina, and in most cases the phenol red was in high concentration. It was pointed out, however, that tubular excretion might not be the necessary mechanism producing these results. They believed that they were as easily explicable on the basis of inward diffusion of water and dye or dye alone at one level of the tubule with subsequent concentration by the reabsorption of water and retention of dye at a lower level. In this view the active function of the tubule is not related to the entrance of the dye but to its retention and the active extrusion of water. They further pointed out that in the kidney of the living animal, *in situ*, it is inconceivable that the concentration of the dye can be increased directly by inward diffusion, since, by reason of glomerular elimination, the tubular fluid already contains it in concentration as high as, or higher than, that in which it exists in plasma water. (It may be noted that this suggestion as to the mechanism of transfer of phenol red must be

considered in the interpretation of double perfusion experiments on the frog's kidney when the renal portal system supplies the test substance; in this case the glomerular fluid contains relatively little of the substance as compared to the renal portal fluid, and conditions for the inward diffusion of the substance are present.) It was suggested that transference of water and phenol red might be accomplished in the normal kidney by filtration across the tubule cells as the result of the capillary pressure. While from histological considerations it seems unlikely that free diffusion occurs across the tubule cells or that the tubules have sufficient structural rigidity to permit inward filtration, there was no direct evidence opposing such possibilities at that time. There was, however, other evidence of a quantitative nature indicating that the excretion of phenol red in the frog as well as in the dog is in part by a tubular process (54, 59). The authors did not believe that their experiments proved the nonexistence of a secretory process in the renal tubule but they suggested that the simple filtration-reabsorption hypothesis of urine formation be retained until more conclusive evidence forced the acceptance of tubular excretion as one of the mechanisms of this process. This viewpoint is evident in other early contributions from Richards' laboratory and was a factor in the production of more critical experiments by the proponents of tubular excretion.

The utilization of histological preparations to evaluate the distribution of substances and their relative concentrations in the various parts of the nephron is an experimental extension of the direct observation of functioning kidneys. In such experiments the test substances are visualized by virtue of their own tinctorial properties or by histochemical procedures. These techniques are subject to an additional criticism as well as those cited in relation to simple observation. The handling of the tissue in its histological preparation may permit a rearrangement, by diffusion, of the concentration relationships existing in the functioning organ. The recent improvements in the preparation of tissues for examination (28) remove, in large part, this objection, but such procedures have as yet added little to our knowledge of these processes. Except for a possible bearing on the excretion of urea in the frog, the chemical determination of concentration relationships between plasma, urine, and various parts of renal tissue is of little aid in the interpretation of the mechanism of excretion of a substance. These techniques are more quantitative than the others in their evaluation of these relationships but the same objections may be brought to bear as in the case of the direct visual examination. We have made a possible exception

considered in the interpretation of double perfusion experiments on the frog's kidney when the renal portal system supplies the test substance; in this case the glomerular fluid contains relatively little of the substance as compared to the renal portal fluid, and conditions for the inward diffusion of the substance are present.) It was suggested that transference of water and phenol red might be accomplished in the normal kidney by filtration across the tubule cells as the result of the capillary pressure. While from histological considerations it seems unlikely that free diffusion occurs across the tubule cells or that the tubules have sufficient structural rigidity to permit inward filtration, there was no direct evidence opposing such possibilities at that time. There was, however, other evidence of a quantitative nature indicating that the excretion of phenol red in the frog as well as in the dog is in part by a tubular process (54, 59). The authors did not believe that their experiments proved the nonexistence of a secretory process in the renal tubule but they suggested that the simple filtration-reabsorption hypothesis of urine formation be retained until more conclusive evidence forced the acceptance of tubular excretion as one of the mechanisms of this process. This viewpoint is evident in other early contributions from Richards' laboratory and was a factor in the production of more critical experiments by the proponents of tubular excretion.

The utilization of histological preparations to evaluate the distribution of substances and their relative concentrations in the various parts of the nephron is an experimental extension of the direct observation of functioning kidneys. In such experiments the test substances are visualized by virtue of their own tinctorial properties or by histochemical procedures. These techniques are subject to an additional criticism as well as those cited in relation to simple observation. The handling of the tissue in its histological preparation may permit a rearrangement, by diffusion, of the concentration relationships existing in the functioning organ. The recent improvements in the preparation of tissues for examination (28) remove, in large part, this objection, but such procedures have as yet added little to our knowledge of these processes. Except for a possible bearing on the excretion of urea in the frog, the chemical determination of concentration relationships between plasma, urine, and various parts of renal tissue is of little aid in the interpretation of the mechanism of excretion of a substance. These techniques are more quantitative than the others in their evaluation of these relationships but the same objections may be brought to bear as in the case of the direct visual examination. We have made a possible exception

any other normal urinary constituent and more than glucose in the phlorizinized frog; this concentration is effected by both the proximal and distal segments; and the degree of final concentration is inversely related to the plasma concentration of urea. From these facts it was concluded that, in the frog, urea is excreted in part by a tubular process (105). The initial approach to the problem is essentially an adaptation to the single nephron of comparative concentration ratios, a method first utilized to advantage on the entire kidney by Mayrs (1924) in the study of uric acid excretion in the bird; all arguments save the localization of function had been previously suggested in the case of urea excretion in the frog (51, 54).[2] In other experiments the product of excretion by a single glomerulus and the number of glomeruli in the renal tissue was taken as the amount of a substance that might be expected to be excreted without the intervention of tubular activity. Such calculations when compared with the measured rate of plasma flow and the total renal excretion led to the conclusion that tubular excretion is not necessary to explain the overall renal elimination of uric acid in the reptile, Eutaenia sirtalis (11). This interpretation was in opposition to the claims of Marshall (52) who, working with Iguana, concluded that the vast majority of uric acid is eliminated by a process of tubular excretion. His claims were based on the belief that the administration of phlorizin completely blocks the tubular reabsorption of glucose, and since it is not secreted, its plasma clearance is equal to the rate of glomerular filtration. It has since been recognized that these premises are essentially correct but that the drug in addition to blocking the reabsorption of glucose interferes with other tubular processes and lowers the rate of glomerular filtration. Because of these reasons Marshall's estimation of the fraction of total uric acid excretion attributable to a tubular process is probably not accurate, but these facts do not invalidate the primary conclusions of the contribution. The variability in the size and functional activity of glomeruli must then be too great for the resultant of the activity of one to be used as even an approxima-

[2] In Marshall's experiments the xylose clearance was used as a measure of the rate of glomerular filtration. It is now known that a small percentage of the filtered xylose is reabsorbed by an active tubular process (88). In the frog this amounts to about 20 per cent (27). In comparison to the quantitative difference between the simultaneous clearances of urea and xylose in the frog this fraction can be neglected in relation to the establishment of the tubular excretion of urea in this animal.

tion of the mean filtration rate of the others contained in the kidney. The importance of the observations on the function of single nephrons in the frog and Necturus cannot be overemphasized in their delineation of the process that characterizes glomerular function, or among other things, in the localization of certain tubular functions to specific segments of the nephron. It would seem, however, that relative to processes of tubular excretion, they have added little evidence of a positive nature that had not been previously adduced by more simple methods.

Credit for establishing tubular excretion as a necessary factor in the renal elimination of certain substances must be accorded Marshall and his co-workers. This advance was characterized by the application of more quantitative methods relating renal excretion and the plasma concentration of the substance under investigation. Working with phenol red Marshall and Vickers (1923) confirmed the observation of De Haan (1922) that this substance does not exist in plasma entirely in a filtrable form.[3] They further showed that when reasonable assumptions are made for renal blood flow in the dog, the amount of phenol red excreted is greater than the amount that reaches the kidney in a filterable form. On the basis of the data then available they considered 5 cc. per gram of kidney per minute a liberal figure for the renal blood flow and a wealth of observations on this function has since justified this belief. A similar conclusion could have been arrived at from the data of De Haan on the rabbit, but this author chose to interpret his data to mean that the formation of glomerular fitrate is accompanied by the filtration of large quantities of plasma protein to which phenol red and other dyes are bound. The filtered protein, in this viewpoint, undergoes subsequent reabsorption with the liberation of the dye for excretion. This hypothesis was untenable at the outset in view of the simultaneous demonstration by Wearn (1922) that the product of glomerular activity is protein free. The third observation of Marshall and Vickers, though less important in the establishment of their hypothesis, is in keeping with it. They demonstrated that when phenol red is injected intravenously into dogs, anuric because of low blood pressure, a large fraction of the injected dye is shortly localized to the cortex of the kidney.

The following year Marshall and Crane (1924) added substantial evidence in favor of the tubular excretion of phenol red in the dog and

[3] It should be noted that De Haan found low values for the free moiety of phenol red in rabbits' plasma (1.25 per cent. as compared to 2.5 to 5.0 found by Marshall and Vickers and 4.8 to 6.7 found by Grollman, 1925). De Haan's error is sufficiently large to somewhat lessen the forcefulness of his argument.

extended the concept to include the excretion of this substance and urea in the frog. The data in the latter two cases were not as convincing quantitatively as was the excretion of phenol red in the dog but seemed acceptable in the light of those data. It was shown that as the plasma concentration of these substances is raised their renal excretion increases but not in direct proportion. This is contrary to expectation for substances that are excreted exclusively by a glomerular process. They offered this curvilinear relationship between plasma concentration and renal excretion as a dependable criterion for the presence of tubular excretion as a mechanism in part responsible for the renal elimination of a substance. They suggested that this relationship followed from the saturation, in the case of phenol red, of cellular colloid which thus limited the availability of material for the active tubular process. The second criterion, the localization of a substance in higher concentration in renal tissue than in plasma or urine, suffers the criticisms mentioned in the beginning of the present section. The curvilinear relationship was the first demonstration of a systematic limitation in this type of renal tubular activity. While the explanation offered by the authors is probably not correct, this criterion has been a valuable aid in the subsequent examination of these mechanisms. In theory this relationship may not be a necessary finding in systems of tubular excretion (see below); actually, however, it has been described in the case of every substance that has been shown to be excreted in part by a tubular process.

Subsequent work showed that the renal excretion of phenol red at low plasma concentrations is an average of seven times that which can be accounted for on the basis of glomerular filtration of free dye (50, 79). Furthermore, it was found that in dogs anesthetized with paraldehyde, the renal extraction ratio is greater than the fraction free dye/total dye of plasma; the former averages about twice the latter at low plasma concentrations (50). This observation acquired added significance from the demonstration that the dye removed from the plasma, or its equivalent, is concomitantly excreted by the kidney (94). The demonstration that the bound moiety of phenol red is available for tubular excretion but not for glomerular elimination is in keeping with the physical properties of such a union since in the latter case there is no disturbance of the free concentration with which the bound dye is in equilibrium (34).

Before the completion of the above investigation on phenol red excretion, the study of the comparative physiology of the kidney had firmly established tubular excretion as a major factor in the formation

of urine (q.v. 53). When the renal function of certain aglomerular marine teleosts was compared to that of their glomerular counterparts, there was evidenced a surprising capacity on the part of the proximal tubule for the excretion of substances by an active tubular process (23, 49, 55). There is little difference in the rate of urine flow in the two types of kidneys and the aglomerular kidney is capable of excreting and concentrating a wide variety of normal and foreign plasma constituents. There are, however, striking differences in the reaction of the aglomerular and glomerular kidney to certain specific substances. The aglomerular kidney is completely unable, or essentially so, to excrete glucose during hyperglycemia or after phlorizin, ferrocyanide (55), cyanol (41), xylose (43), sucrose (96), inulin (73, 77); and protein either normally or after the administration of $HgCl_2$ (6). It was further shown that the efficiency of excretion of injected substances (phenol red (8, 56); creatinine (56)) is diminished as the amount of the injection is increased. This is not due to a systematic variation in absorption but to the characteristic relationship between plasma concentration and tubular excretion (86, 87). These facts cannot be explained on the basis of diffusion as suggested by Richards and Barnwell to explain the entrance of phenol red into the tubule lumen of the more complex nephron of the frog. Such nonspecific diffusion of water and solutes is ruled out by the specificity of the processes, the relationship between plasma concentration and rate of elimination, and the finding of a single cell type in the anatomical makeup of the tubules (21, 31). It was also shown that the secretion pressure the tubules are capable of developing is greater than that existing simultaneously in the aorta (7). This definitely rules out filtration across the tubular membrane as a factor in the formation of urine in these kidneys. It is not known whether the excretion of water by the aglomerular nephron is a primary and independent process; the fact that high plasma concentrations of certain substances which are actively excreted (magnesium, sulphate, etc.) increase the rate of water excretion (9, 10) suggests that this is not true. This interesting possibility must await further investigation. It was also evident from many investigations that tubular excretion is important in the formation of urine in many types of glomerular kidneys. This is particularly apparent in those classes of vertebrates that are compelled, because of environment, to conserve water and yet have a limited capacity for its renal tubular reabsorption, i.e., marine fishes, reptiles, and birds (53).

Further evidence that the cells of the proximal tubule are at least potentially capable of actively depositing material in their tubule lumen was adduced by Chambers and Cameron (1932), and Chambers and

Kempton (1933). These investigations showed that fragments of the proximal tubules of the chick embryo mesonephros can be maintained in a viable state in tissue culture for long periods of time. Under proper conditions the ends of such a fragment close and the lumen becomes distended with fluid as the result of the activity of the cells comprising its wall. When phenol red, or one of a number of sulphonated dyes, is placed in the media it is accumulated in the cyst fluid in concentrations many times that of the surrounding media; in fact, the media immediately adjacent to the cyst may become completely cleared of the dye. To this evidence of an active process was added the fact that factors which inhibited cellular metabolism are capable of reversibly inhibiting the transfer of phenol red. The agents which have been shown to produce this inhibition are low temperature (15), cyanide, hydrogen sulphide, oxygen lack (16), and sodium iodoacetate (5). The effect of the latter agent is counteracted to a large extent by the addition of sodium lactate, pyruvate and succinate. The essentials of these observations have been extended to the chick metanephros and the metanephros of several mammals including man (12).

A general criticism of the methods discussed so far is that they are only qualitative or semiquantitative appraisals of the resultant of tubular excretion and give no definite knowledge of these activities in the normal animal; nor do they give any clear indication of, or method of attack on the mechanism of transfer or its normal cellular limitations. To obtain information useful for these purposes it is necessary to have available a method that will give precise information on the resultant of tubular activity in the normal glomerular animal. Such a method of attack is available at the present time. It lies in the ability to measure the amount of any substance that is filtered by the glomeruli per unit time by the simultaneous determination of the diffusible concentration of that substance in the plasma and, by means of the inulin plasma clearance, the rate of glomerular filtration. The product of these two is equal to the amount of the substance filtered per unit time. If reabsorption can be excluded, the difference between this quantity and that concurrently excreted can be attributed to the process of tubular excretion.[4] That the inulin clearance can be safely used as an accurate

[4] The general statement needs qualification in certain specific cases. Chambers and Kempton (1937) have shown that the excretion of neutral red, which cannot be entirely accounted for on the basis of glomerular filtration, is in part by the tubules. One factor operating in this case is not a specific tubular process but a difference in hydrogen ion concentration which is an effective determinant of the direction of the dye's diffusion.

measure of glomerular filtration rate is indicated by evidence that has been obtained in a wide variety of vertebrate kidneys (44, 64, 72, 74, 77, 78, 81, 83, 84, 93, 98, q.v. 99).[5]

Utilizing this technique the renal excretion of a wide variety of substances, many of which are excreted in part by a tubular process, has been studied in the normal animal. It has been possible in some cases to obtain precise information which permits a tentative analysis of certain aspects of the cellular mechanisms involved. It is interesting to note that certain characteristics of tubular excretion discussed below have been found to be present in the case of some processes of tubular reabsorption. This section of the discussion will be made sufficiently general to include these aspects of the resorptive processes.

On the mechanism of tubular excretion. In seeking the physiological basis for these processes one is led to the following considerations, which are presumed to apply to both the glomerular and aglomerular kidney:

1. The cellular processes responsible for the transfer of a solute from the blood to urine, or vice versa, are orderly ones which proceed in accordance with the principles of thermodynamics and the kinetics of chemical reactions, the direction of the transfer being determined by the organization of the tubule cells. For convenience the sequence of events which results in such transfer will be designated the mechanism of tubular excretion or reabsorption, or, more briefly, the mechanism of transfer. By definition this term excludes all extra-cellular processes, such as diffusion or chemical reaction in the peritubular interstitial fluid and urine.

[5] The importance of this advance is evidenced by the many attempts at its experimental solution prior to the establishment of the inulin clearance as a valid measure of filtration rate. Many other substances had been suggested which were later shown to be reabsorbed or secreted in one or more vertebrate kidneys. The first systematic study of importance was that of Mayrs (1924) in relation to the renal excretion of uric acid by the chicken. He reasoned that, since the rates of excretion of creatinine, sulphate and phosphate, relative to their plasma concentrations, were in the same order of magnitude, they were excreted by a predominantly glomerular process. Uric acid excretion, when observed simultaneously with these, was sufficiently high to suggest in addition a process of tubular excretion; this was in keeping with the high absolute concentration ratio. It is now known that Mayrs' reasoning was essentially correct (29, 52, 84, 85). Many of the other substances suggested, while they do not satisfy the requirements for the precise measurement of filtration rate, are closely proportional to it, and in some cases can be corrected to this value by an experimentally determined factor.

2. Where the mechanism of transfer increases the free energy of the system, this energy is presumed to be made available entirely by local cellular metabolism. To what extent the energy expenditure is proportional to the quantity of solute transferred, whether this energy may be diverted into some other operation in the absence of the solute, whether it is derived from processes that proceed wholly independent of the mechanism of transfer, or whether it is liberated in consequence of the presence of the solute, cannot be ascertained.

3. In some of the reactions involved in the mechanism of transfer, the transferred solute as such, or in combination, will be one of the reactants. Other reactions may not be so characterized (e.g., the energizing reactions). Each of the reactions in the former group may be said to be made up of two reactants or elements, the substance being transferred and the cellular element. All such cellular elements may be designated as the cellular component of the system and the substance transferred as the exogenous component.

4. It may safely be inferred that the cellular component is a more or less stable feature of the organization of the cell and does not arise *de novo* on the presentation to the cell of the exogenous component of the system.

5. Except where there is evidence to the contrary, it is assumed that a cellular component of any system is to some degree specific with respect to the solute transferred; and that the mechanism of transfer is identical in all species in which the process of transfer is demonstrable.

6. A cellular component may be common to the transfer of more than one substance. The extent to which more than one substance shares a cellular component is, theoretically at least, variable. The cellular component in its entirety, or only certain of its elements, may be common to the reactions of transfer of the two substances.

7. The mechanism of transfer is limited in respect to the quantity of any one solute which it can handle per unit time, and when two solutes are transferred simultaneously by a common mechanism, one will displace the other to a greater or lesser degree dependent upon their respective affinities for the common elements in each of the cellular components.

8. A primary determinant in the rate of transfer of a substance, whether it occupies the cellular component alone or is, with another substance, in simultaneous competition for it, is the concentration of that solute in the peritubular interstitial fluid in the case of an excreted substance, and in tubular urine in the case of a reabsorbed substance. In

both cases the concentration in the fluid of deposition must also be considered a potential determinant limiting the rate of transfer, which may or may not be practically significant under physiological conditions.

9. The non-specific characteristics of a substance such as lipoid vs. water solubility and molecular volume are of no demonstrable importance in determining the rate of cellular transport unless the substance is in a colloid state or some molecular aggregate which makes it unavailable for transfer by the cellular mechanism.

Without discussing the evidence in detail, we may briefly summarize the basis for the above:

1, 2, 3. These need no specific experimental substantiation since they are derived from accepted general principles or are in the nature of specific definitions of the processes under consideration. Certain conclusions, however, may be drawn in relation to the energizing reactions. It is conceivable that under certain experimental conditions these reactions may impose limitations on the active transfer of a substance. On the other hand, in normal circumstances, there is adequate evidence to rule out these reactions as limiting the processes discussed herein. This evidence consists in the uniform presence of transfer maxima and the demonstrated lack of influence, in certain of these systems, of the diffusion gradient or concentration ratio upon the rate of transfer. (Phenol red excretion in the aglomerular fishes (87), glucose reabsorption in the dog (91).)

4. The concept of stability in these systems is derived from the quantitative reproducibility of the resultant of their activities over long periods of time, the specific details of which are contained in the articles referred to below. Such quantitative stability seems most easily explicable on the basis of continuity of existence of the cellular component in the absence, as well as in the presence, of the exogenous component.

5. The specificity of the mechanism by which various solutes are transferred across the tubules is indicated by numerous facts. Glucose is specifically reabsorbed by the tubules of all glomerular kidneys, as is xylose, and sucrose but to a much smaller extent and inulin not at all (q.v. 93); in this instance the nature of the carbohydrate obviously determines whether reabsorption will or will not take place and if so to what extent. Phenol red is excreted by the aglomerular tubules and by the tubules of all glomerular kidneys where appropriate examination has been made; only two other exogenous substances, diodrast and hippuran (25, 47, 101) and one endogenous substance, uric acid in the

chicken (52, 85) and reptile, (52) are known, which are excreted as copiously. This highly developed capacity to excrete a particular substance indicates some special feature in the functional organization of the tubule cells. Creatine is excreted by both the glomerular and aglomerular tubules of the fishes (55, 63) but not by mammals (62); creatinine is excreted by the aglomerular and glomerular tubules in the fishes (56, 76, 77, 86) and by the glomerular tubules of the chicken (84) and some mammals, man (80) and apes (100), and not in others (dog (74, 78, 103), sheep (83), rabbit (44), seal (98)); urea is excreted by the tubules of some fishes (56) and of the frog (51, 54, 104), but not in Necturus (104), the chicken (65), or mammals (18, 44, 82, 83, 93), and it is actively reabsorbed in the elasmobranch fishes (97); uric acid is excreted by the tubules in the birds (52, 85) and reptiles (52), but it is reabsorbed in the mammals (q.v. 99). This great diversity in the manner in which various solutes are handled by the renal tubules in various vertebrates precludes the possibility that the essential processes of tubular excretion and reabsorption are of such a nature as to transfer large numbers of unrelated solutes non-specifically and requires instead that a more or less specific process be postulated for the transfer of each one.

In arriving at our postulate that the mechanism of transfer for a particular solute is identical in different species, we proceed first from the broad generalization that the renal tubules are strictly homologous in all vertebrates; although, recognizing the distinct ontogenic difference between the mesonephros and metanephros, there is no good reason for supposing that function in these two organs is not homologous. It may be that the glomerular tubule of the sculpin excretes inorganic sulphate although the evidence is not strong and other interpretations of the data are possible (32, 57). With this possible exception, in every case where a test has been made, the aglomerular tubule has shown its capacity to excrete a substance which has been shown to be excreted by a tubular mechanism in the glomerular tubules of the vertebrates. It may be argued from this, that where a mechanism for the tubular excretion of a substance exists in the vertebrate kidney it is there as the result of the persistence of the primitive characteristics of the protovertebrate aglomerular tubule (58).[6] In this view the presence or absence of various

[6] We do not suggest that the aglomerular kidney is a primitive organ closely related to that of its protovertebrate ancestors. The aglomerular state is evidence, in these forms, of a high degree of specialization which has entailed the secondary loss of previously acquired glomeruli (for discussion see Marshall and Smith, 1930).

mechanisms of transfer in these vertebrates must be sought under the broad principles of genetics. Though it is conceivable that specific mechanisms having the same net result might have arisen more than once in vertebrate evolution and might, therefore, have a different fundamental nature, it would seem superfluous at the present time to abandon the principles of uniformity and persistence until we were forced to on the basis of experimental evidence.

6. Although this may be accepted in principle, the experimental facts relating to this are discussed under the following two items.

7. That these mechanisms of transfer possess internal limitations is well established. In every example of tubular excretion examined to date, it has been demonstrated that the rate of tubular excretion is not related in a linear fashion to the concentration of the solute in the plasma. The first system examined for these limitations in a quantitative manner is that responsible for the tubular excretion of phenol red in the dog (79), a system quite characteristic of this type of renal activity. It was demonstrated that, as the plasma concentration of free dye is increased from 0.02 to 0.4 mgm. per cent the rate of tubular excretion increases in what is roughly a linear manner; as the plasma concentration is further elevated the rate of tubular excretion increases but not linearly, and approximates a maximal rate at plasma concentrations of from 5.0 to 10.0 mgm. per cent free dye. It appears in all instances that the rate of transfer ultimately reaches some constant maximal value, i.e., the mechanism of transfer behaves as though it were saturated with respect to the solute. This maximal rate of transfer from blood to urine has been demonstrated in the tubular excretion of phenol red in the toadfish and goosefish (87), chicken (64), and man (30, 101) as well as in the dog; of creatinine in the dogfish (76, 77, 90), toadfish (86) and chicken (84); of diodrast and hippuran in man (101). It has also been demonstrated in the tubular reabsorption of glucose in the dog (91) and vitamin C in man (66).

8. This condition is one of the more important ones listed above; particularly since a decision as to its correctness must be arrived at before a consideration of the fundamental nature of the cellular limitations of these systems is possible. Its probable correctness may be deduced from 1 and 4. If the cellular component is a stable property of the cellular organization and the reactions of transfer proceed in accordance with the principles of the kinetics of chemical reactions, it must follow that the concentration of the exogenous component of the system at the proximal side of the sequence of reactions is a determinant in the initiation and progress of these reactions. The quantitative

reproducibility of the resultant of the activity of the mechanism of transfer in the case of one or two exogenous components in relation to the concentrations at the proximal side of the sequence furnishes adequate substantiation of this deduction. That a particular mechanism may be responsible for the transfer of several solutes and consequently, that solutes may compete for this mechanism has been demonstrated for the system responsible for the transfer of phenol red, diodrast, hippuran, iopax, neo-iopax, and skiodan in man (101, 102), and that responsible for the tubular reabsorption of glucose and xylose in the dog (88, 91). This interference is of a systematic quantitative nature which may be related to the concentrations under discussion. Though the point and nature of the interference may be different in each case they establish the principle that certain substances may occupy the cellular component to the exclusion of other substances, the extent of this interference depending upon the specific nature of the exogenous component and its concentration at the site of the initiating reactions. If cellular storage takes place preliminary to, or in the process of its tubular transfer, the localized solute must be considered to be in dynamic equilibrium with the concurrent plasma concentration.[7] This is evidenced by the quantitative reversibility of many systems of transfer in relation to the elevation and lowering of the plasma concentration. In certain systems, at least, such storage cannot be shown to exist. The amount of localization of phenol red in the dog's functioning kidney (94) is scarcely more than can be accounted for on the basis of the contained urine. This is in keeping with the inability to demonstrate cellular localization during the transfer of phenol red by the cells of the chick embryo mesonephros (15).

There is little evidence available on the effect of the concentration in the fluid of deposition on the transfer of a solute. The phenol red

[7] In arriving at this conclusion we have been forced to disregard the experiments of Elsom, Bott and Walker (1937). They determined simultaneously on anesthetized rabbits the plasma clearances of hippuran or phenol red and the renal blood flow (4, 75). It was found that, contrary to Sheehan (1936), the excretion rate of phenol red was on the average two and in one case four times the rate of delivery of this substance to the kidney. In experiments with consecutive periods this was true even though the plasma phenol red was rising. They concluded from their experiments that the phenol red excreted in an experimental period must have been in part stored in the renal tubules prior to its beginning. In view of the evidence opposing such a concept of storage, and in the absence of a correlation in the data between plasma concentrations and phenol red clearances, we feel that these experiments were complicated by an unrecognized experimental difficulty.

system in the toadfish (87) has been shown, by direct evidence, to be independent of this factor[8] and this is supported by the presence of a maximal rate of transfer in the glomerular nephrons of other systems. Whether the increase in the tubular excretion of creatinine with increase in urine flow, observed in the toadfish (56, 86), is due to a complication introduced by the movement of water in the same direction as that of the transfer or is due to a lowering of the concentration in the fluid of deposition, cannot be determined with the evidence available.

9. It is obvious that unless a solute can penetrate the cell membrane for action upon by a cellular mechanism, active transport of such a solute is not possible unless the active process is initiated at the cell-interstitial fluid interface. The evidence on the renal tubule (cf. 42) is not extensive but would seem to be in keeping with studies on other systems of active transfer. One may show some relationship between lipoid solubility or molecular volume and the permeability of the tubular membrane for a substance in certain series of solutes. Invariably, however, when there is included in such a series a substance handled by one of these specific cellular processes, the rate of transfer is of an entirely different order of magnitude.

With such complexity, it is beyond the range of possibility to erect an hypothesis at the present time which would pretend to be a complete description of any mechanism of transfer, and particularly to include that portion of it wherein energy is made available and specifically applied. Nevertheless there are certain striking features that suggest the nature of certain of the limitations contained in these systems. These features are the reproducibly quantitative relationship between the concentration in the initial fluid and the rate of transfer, the latter of which is in all cases characterized by a maximal rate. If we view the reactions of transfer as a sequence of reactions of unknown number, proceeding in accordance with principles of chemical kinetics, it is possible to erect an hypothesis that will contain the major limitations which these systems manifest.

[8] It is interesting to note that in the toadfish the cell membrane acts as a barrier to the free diffusion of the dye in the same direction as it is normally transferred actively (87). This was demonstrated by finding that when the plasma concentration of free dye is higher than the concentration within the tubule lumen there is no increase in the rate of excretion of the dye as compared with a previous period when the concentration relationships were reversed. It would seem from this observation that the tubule cells of the toadfish are actually impermeable to phenol red except through the active mechanism of transport.

In the case of an excreted substance one may assume, first, that in the sequence of reactions that result in its transfer, the solute enters into reversible combination with some cellular element which is present in a constant but limited amount, and second, that the decomposition of this complex limits the further progress of the solute toward the tubule lumen. Thus there are required two consecutive reactions, as follows,

$$A + B \rightleftarrows AB \rightarrow T_s + B \tag{1}$$

where A is the solute at the proximal side of the reaction (in the interstitial fluid around the tubule cells), B is the cellular element, AB the complex formed reversibly by these two, and T_s the solute on the distal side of the limiting reaction. In order to arrive at a maximal rate of excretion under these circumstances, the second reaction must be a first order process, its rate slow in relation to the rate of attainment of equilibrium in the first. It is theoretically possible that in the case of certain solutes actively transferred, the rate of the second reaction $AB \rightarrow T_s + B$ is faster than the attainment of equilibrium in the first. Under these conditions the rate of transfer of a substance would be linearly related to its plasma concentration (see xylose reabsorption, 88). These equations completely neglect the fact that the transfer process involves the expenditure of energy; they do imply that it is not a limitation in the energizing reactions which limits the rate of transfer in the normal animal. It is implicit in the first reaction, $A + B \rightleftarrows AB$, that this step in the tubular transfer is effected at the expense of the free energy of the three reactants. However, the designation of the second reaction as a first order process does not preclude the possibility that this may be complex and involve an increase in free energy.

In pursuing the quantitative implications of this hypothesis it is apparent that if total B (i.e., as B and AB), is constant and limited in amount, it matters little whether it be assumed that we are dealing with stoichiometric processes involving concentrations in a monophasic system, or with certain absorptive processes in a polyphasic system (40). For simplicity in treatment, however, we have assumed the former of these possibilities so that both of these reactions may be described in accordance with the law of mass action. The working equation so derived[9]

$$K = (a - T_s/V)\left(\frac{T_m - T_s}{T_s}\right) \tag{8}$$

[9] We may therefore write, for the first reaction;

$$K = \frac{(A)(B)}{(AB)} \tag{2}$$

relates in a quantitative fashion the arterial plasma concentration, a, the rate of tubular excretion T_s, and the maximal rate, T_m. Of the terms in the equation, a and T_s can be measured directly; V can be closely estimated in appropriate instances; K and T_m must be calculated from the data. However it should be noted that T_m, the maximal rate of tubular excretion, can in some cases at least be experimentally approximated. It is apparent that the validity of the test of this relationship depends upon the range of T_m/T_s and of $(a - T_s/V)$ in the comparison with experimental data. For convenience the units of

---

where (A), (B) and (AB) are equilibrium concentrations at the site of the reaction and K is the equilibrium constant. Designating total B as b, since the sum of the concentrations $(B) + (AB)$ is constant:

$$K = \frac{(A)\ (b - (AB))}{(AB)} = (A)\left(\frac{b}{(AB)} - 1\right) \tag{3}$$

In relation to the second reaction, $AB \rightarrow Ts + B$, consider the general case,

$$k(AB) = T_s \text{ or } (AB) = T_s/k \tag{4}$$

where Ts is the amount of substance secreted per unit time at various values of (AB); and the special case where transfer is at its maximal rate, i.e.,

$$k(b) = T_m \text{ or } (b) = T_m/k \tag{5}$$

where T_m is the maximal rate of transfer as would be the case when total $B = AB$.

Dividing (5) by (4);

$$(b)/(AB) = T_m/T_s \tag{6}$$

Substituting in (3)

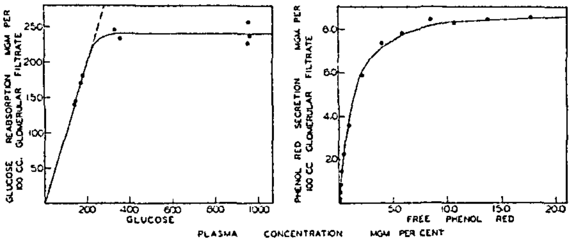
$$K = (A)\left(\frac{T_m - T_s}{T_s}\right) \tag{7}$$

The concentration of A in the venous blood leaving the secretory system is equal to the arterial plasma concentration, a, minus the amount secreted per unit time, T_s, divided by the plasma flow per unit time, V, i.e., $(a - T_s/V)$. When the diffusion gradient from plasma to the site of the initial reaction is not large in relation to (A), this term may be taken as its equivalent, and when V is very large in relation to T_s this term reduces to (a). Under any circumstance where the removal of A from tubular plasma is great it is apparent that this approximation may introduce a distortion since various parts of the secretory system will be presented with the solute in different concentrations, also the magnitude of the diffusion gradient which cannot be experimentally evaluated, becomes significantly large in relation to the term $(a - Tr/V)$. Errors from this approximation will be, under these conditions, most marked at low plasma concentrations. With this substitution equation (7) becomes

$$K = (a - T_s/V)\left(\frac{T_m - T_s}{T_s}\right) \tag{8}$$

measure are taken as milligrams, minutes, and the standard of volume reference 100 cc.

It is possible in some systems to obtain experimental data of sufficient completeness and precision to warrant comparison with the predictions of equation 8. These show that the relationship between the plasma



1a 1b

Fig. 1a. The tubular reabsorption of glucose in the normal dog. The dots are observations obtained in a single experiment. The broken line and its solid extension indicate the quantity of glucose delivered to the reabsorptive system by glomerular filtration. The solid line is calculated from equation 8 taking $K = 0.2$ and $Tm = 240$ (c.f. 91) at plasma concentrations above 300 mgm. per cent. Tr is within 99 per cent of Tm.

Fig. 1b. The tubular excretion of phenol red in the normal dog. The dots are observations obtained in a single experiment. The line has been calculated from equation 8, taking $K = 0.92$, $Tm = 8.95$, and $V = 12$ (the plasma water going to the tubules is four times the amount of glomerular filtrate (103)) and at the lower concentrations of phenol red the free fraction is 0.3. The latter factor increases the apparent volume of distribution of the dye in plasma water about three-fold. Our previous observation of an absolute maximal rate of tubular excretion reached in the neighborhood of 6 mgm. per cent free dye (79) is changed by correcting the plasma inulin clearance to plasma water inulin clearance (see 64). With this correction the rate of tubular excretion is still rising slowly at this concentration. At 15 mgm. per cent free dye the fraction $\frac{Ts}{Tm}$ is equal to 0.95.

concentration and the rate of tubular excretion and the maximal rate is satisfactorily described by this equation in the systems responsible for the tubular excretion of creatinine in the dogfish (cf. 90) and chicken (84), and of phenol red in the dog (79, see fig. 1b). When applied to tubular reabsorption a similar close correlation has been found for

glucose in the dog (91, see fig. 1a) and vitamin C in man (66).[10] Here it is assumed that the physiological orientation of the system is reversed, so that (A) is the equilibrium concentration in tubular urine. In the other systems mentioned above, the comparison must be of a more qualitative nature due to deficiencies in the existing data or because of experimental limitations in the biological material.[11] Such comparisons as are possible in these systems justify the tentative acceptance of our hypothesis, at least as a working basis for their further examination.[12]

The case of two solutes in simultaneous competition for a common cellular component has been examined in two instances. The first by Smith, Goldring and Chasis (1938) for the systems responsible for the transfer of phenol red, diodrast and hippuran in man, and the second by us for the systems responsible for the reabsorption of glucose and xylose in the dog (88, 91). In the former instance, the tubular excretion of phenol red at constant and low plasma concentration is reversibly

[10] In the case of reabsorptive systems the term $(a - Tr/V)$ relates arterial plasma concentration, rate of reabsorption and volume of glomerular filtrate. It is equal to the concentration at the distal end of the reabsorptive system (presumably proximal tubule) if there has been no water reabsorption. It seems likely that there is a significant amount of water reabsorbed in this segment (71, 89) but unless the test substance itself affects the process of water reabsorption, the increased concentration will be some multiple of $(a - Tr/V)$ which automatically becomes incorporated in the constant K (q.v. 88, 91).

[11] They arise from: 1. The necessity of examining mean relationships derived from the massing of data from many individuals; it being impossible to collect all the necessary data in single well controlled experiments. 2. The inability to use $(a - T_s/V)$ as a close approximation of (A) either because tubular excretion effects what seems to be nearly complete removal of the solute or because of insufficient knowledge of the renal blood flow. 3. The inability to examine the entire relationship because of high endogenous values for the plasma concentration or excretion of the solute or because high concentrations of the solute are physiologically active and produce an additional variable. 4. A quantitative relationship between the amount of a solute excreted by glomerular filtration and that excreted by tubular activity which makes it difficult to examine the latter with precision; this is particularly apparent at high plasma concentrations of the solute. 5. The presence of other adventitious circumstances. The non-reversibility of the system responsible for the tubular excretion of creatinine in man (80) is one of these. At present this seems to be due, not to a change in the function of the tubules during the course of an experiment, but to some change in the creatinine itself, effected extrarenally (92).

[12] The hypothesis we suggest does not contain an explanation of the influence of urine flow on the tubular excretion of creatinine in the toadfish (86). For the present, however, we accept it as the basic limitation and await further experimental evidence which may permit its modification.

depressed, with essential completeness, by raising the plasma concentration of diodrast or hippuran to a plasma concentration that results in its maximal rate of tubular excretion. The relationship between the increase in plasma iodine and the depression of phenol red excretion has been considered sufficiently close to the predicted relationship to warrant a tentative acceptance of a B substance common to both systems in accordance with equations 1 and 8. The inability to approximate (A) by $(a-T_s/V)$ in these experiments prevents a more rigid examination of the data. In the xylose-glucose system the results fully bear out the quantitative predictions of the hypothesis, the most striking finding being that the elevation of the plasma glucose, to a concentration just sufficient to saturate the glucose reabsorption system, completely and reversibly blocks xylose reabsorption. This circumstance was predictable from an evaluation of the constants in each of the separate systems.

It does not seem reasonable to suppose that the limitations of all renal systems of transfer will be reducible to those suggested here. The substances discussed above are either non-electrolytes or are handled by virtue of the specific properties of their anion or the undissociated molecule. Variations in plasma concentration in the experimental range do not result in a disturbance of the general electrolyte pattern of the plasma. Furthermore, it is highly probable that these systems are characteristics of the proximal tubule, a circumstance that might in part lead to their uniformity in the type of limitation. At present the usefulness of this hypothesis resides in its ability to treat certain features of tubular activity in a systematic and in some cases a quantitative manner; this with respect to the handling of single solutes by a mechanism of transfer and the predictions concerning the resultant of activity in systems where two substances are in simultaneous competition for a common cellular component. With these considerations it becomes apparent that the hypothesis has its primary value in the logical direction, by experiment, of the further examination of these systems.

Physiological importance of tubular excretion. It is apparent from the previous sections that the excretion of substances foreign to the organism has played a major rôle in the development of the concept of tubular excretion. It must not be concluded from this fact that this type of renal activity is unimportant in the normal economy of the organism in at least certain classes of vertebrates. From the standpoint of comparative physiology, it must be supposed that these mechanisms become more important in those forms that are char-

acterized by poor glomerular development, becoming paramount in those that are completely aglomerular. A more extended discussion of this relationship is available in the recent review of Marshall (1934) and the relationship between glomerular development and the evolution of water regulation in the vertebrates and environment is fully discussed in the writings of Marshall and Smith (1930) and Smith (1932).

The absence of mechanisms of tubular excretion would have made difficult or impossible the reduction in the rate of glomerular filtration characteristic of the marine teleosts, reptiles, and birds. This conclusion is not based upon teleological reasoning but upon the experimental data available from these forms. A most striking example of this is found in the excretion of uric acid, the main nitrogenous end product of protein metabolism, in the bird and reptile. It has been pointed out (85) that in the absence of tubular excretion the plasma concentration of uric acid would have to be elevated some ten to sixteen times in order to maintain nitrogen equilibrium. However, the known properties of uric acid, particularly the insolubility of its salts, would lead one to believe that some advantages may accrue from maintaining its plasma concentration at the lowest possible level. The alternate expedient would be to increase the rate of glomerular filtration to a like degree, but a ten- to sixteen-fold increase in filtration rate would entail a corresponding increase in the reabsorptive activities of the renal tubules and the cloaca. Such an increase in filtration rate may be held to be incompatible with both the poor glomerular development and the limited osmotic capacities of these kidneys. It may therefore be argued that the development of a highly efficient tubular mechanism for the excretion of uric acid in the bird and reptile serves to maintain a low plasma concentration of uric acid in spite of, or in association with, relatively poor glomerular development and a limited capacity to elaborate an osmotically concentrated urine.

In some of the other vertebrates tubular excretion also plays a major rôle in the renal elimination of the end products of metabolism, though there it is more an incidental than necessary function (as urea excretion in the frog). There would seem to be little advantage or necessity for the operations of tubular excretion in these forms (i.e., fresh water teleosts, elasmobranchs, aquatic amphibia and mammals). These have available an abundance of water for the elimination of waste substances or have means, other than a limitation of glomerular filtration, to satisfy the demands of their environment for the conservation of water. However, the potentialities of mechanisms of tubular excretion

for the elimination of substances from the organism are sufficiently impressive to warrant consideration from a slightly different viewpoint.

There is adequate evidence for us to conclude that certain mechanisms of tubular excretion are capable of completely removing, or essentially so, specific substances from all the plasma delivered to the kidney (uric acid and phenol red in the bird (64, 85), diodrast and hippuran in man (101)). The importance of such a capacity is greatly enhanced by the large magnitude of the renal blood flow, which in man is about 30 per cent of the cardiac output (see below). It is obvious from these considerations that it would be possible in man, through the intervention of tubular excretion, to maintain at extremely low concentrations in the body substances which, being continuously formed, had a deleterious effect upon the organism, were such substances handled at a rate equal to that of diodrast. We have no knowledge of evidence favoring or opposing the existence of such mechanisms in the normal animal; if they be present, some of the symptomatology characteristic of impairment of renal function may arise from the absence of their normal operation. The answer to this question must await the further identification of those substances which make up the unknown portion of the urinary constituents.

This suggestion should not be taken to mean that the presence of an active tubular mechanism capable of excreting a foreign substance necessitates the conclusion that in the normal animal, in the absence of the foreign substance, the mechanism is concerned with the excretion of some normal plasma constituent. These potentialities of the renal tubule may be characteristics of the tubule cells which are not purposeful in nature. They may be considered to be the results of incidents in the cells' genetic history and, not being deleterious to the organism as a whole, have persisted as part of the cells' functional makeup.

Measurement of tubular excretory mass and effective renal blood flow. Certain studies of recent date have been concerned with the use of these mechanisms in physiological investigations rather than the evaluation of the processes themselves. These investigations have made two important additions to the armamentarium of the renal investigator. They are the indirect measurement in normal man of the effective renal blood flow and the mass of renal tubular tissue (Smith, Goldring and Chasis, 1938; Chasis, Ranges, Smith and Goldring, 1938). We will confine ourselves here to a simple presentation of the concepts and some comments which relate them to the second section of this review.

If a substance is not synthesised by the kidney, its rate of renal excretion has an upper absolute limit which is equal to the amount brought to the organ by its blood supply. The excretion of material stored in the kidney at previous higher plasma concentrations, if this can be shown to be a factor, can be circumvented by making the experimental observations at constant plasma levels of the solute. Since all the blood supply to the kidney is eventually distributed to the renal tubules, it is theoretically possible that all of a contained solute may be removed from the blood as it traverses this tubular tissue, or at least all that traverses secretory tissue. The plasma clearance of such a substance (i.e., UV/P; milligrams excreted divided by the plasma concentration in milligrams per cubic centimeter) will then be equal to the volume of plasma going to secretory tissue. If the substance is not contained in the red blood cells then the quotient of its plasma clearance and the plasma fraction of whole blood will equal the blood flow to secretory tissue.

It has been suggested that in man diodrast at low plasma concentrations is handled in this manner and its whole blood clearance has been called equal to the *effective blood flow* to the kidney. Effective, as opposed to absolute, in that this figure will not contain that moiety of the renal blood supply which is distributed to non-secretory tissue. The main experimental facts that have been utilized to support this contention are: the absolute value of the diodrast clearance and the fact that there must be a significantly large increase in the plasma concentration before the rate of excretion loses its direct proportionality to the plasma concentration. The average effective renal blood flow in normal resting man ($S.A$ 1.73 sq.m.) by this method, has been shown to be 1384 cc./min., approximately 30 per cent of the cardiac output (35); it is not conceivable that the actual total renal blood flow is significantly higher than this value. The direct proportionality between the plasma concentration and renal excretion over a wide range of the former is excellent evidence that there is present a limitation external to the mechanism of transfer operating in the production of this result.

It is possible that disease or the introduction of an experimental variable will lower the diodrast clearance at low plasma levels by lowering the capacity of the tubules to transfer this substance and thus invalidate its use as a measurement of renal blood flow. There are two methods that are available to evaluate such a possibility. The presence of a normal transfer maximum or one that is not greatly reduced is

ample evidence that a low diodrast clearance at low plasma concentration is due to a low blood flow and not to an interference with the mechanism of tubular excretion. The second method makes use of the finding that phenol red and diodrast are handled by a common tubular mechanism, and consists in determining their clearances simultaneously at low and constant plasma concentrations. The limitation in the excretion of diodrast under these conditions is the rate of delivery to the tubules, whereas the limitation in the excretion of phenol red is, in terms of our hypothesis, the availability of the cellular element, *B*, common to both systems of transfer. The absolute and relative excretion rates of these substances indicate that diodrast will appropriate *B* in proportion to its delivery to the tubules displacing phenol red and hence lowering its excretion rate proportionately. At constant plasma concentrations of the two substances any change in the amount of diodrast delivered to the excretory mechanism must be through a change in the rate of blood flow to the kidney. Therefore, an inverse relationship between the diodrast clearance and the phenol red/diodrast clearance ratio may be accepted as the normal relationship with changes in the renal blood flow. On the other hand, if a diminished capacity to excrete the diodrast is effected by an experimental variable, this relationship will not hold. The proportional lowering of the phenol red clearance will be greater than that of diodrast. This follows from changing the limitation on diodrast excretion from the availability of the substance to the tubules to a limitation within the system of transfer. There will result, in this circumstance, a lowering of the phenol red excretion due to the curtailment, by the experimental variable, of the potentialities for its excretion, and due to a further displacement of phenol red from the system of transfer by the iodine compound. The diodrast clearance and the phenol red/diodrast clearance ratio will then move in the same direction. (See action of phlorizin (19).)

The concept of *tubular excretory mass* follows from the demonstration of a maximal rate in the tubular transfer of many substances. This maximal rate, obtained at high plasma concentrations of the solute, is due to a cellular limitation and as such, is independent of blood flow and plasma concentration if these be above certain minimal values. Since it may be accepted that the ability to transfer a substance is a more or less fixed property of the cell, then the value of the maximal rate should be proportional to the number of cells capable of performing this function; hence the concept of excretory mass in the case of an excretory system. Any substance whose active tubular transfer is

characterized by a maximal rate may be used in this manner, and each substance will have its normal value. If a reabsorptive maximum is used it will not include aglomerular nephrons if such exist as functioning units. A reduction in this figure, as compared to the normal, may occur by the progressive loss of functioning cells or by a change in the functional capacity of the existing cells to handle the test substances. In either case there would be a reduction in the functional *mass* in proportion to the reduction of the transfer maximum. For the evaluation of this figure in man, Smith and his co-workers have suggested diodrast or hippuran because in each case a high fraction of the totally excreted substance is by a tubular process, a circumstance which increases the accuracy of the determination. The value of this concept in the study of the kidney must be decided upon after application to further material. This measurement would not be expected to yield information on the functional state of tubular tissue not concerned with these activites, i.e., parts other than the proximal segment.

REFERENCES

(1) Atkinson, M., G. A. Clark and J. A. Menzies. J. Physiol. **55**: 253, 1921.
(2) Bainbridge, F. A., J. A. Menzies and S. H. Collins. J. Physiol. **48**: 233, 1914.
(3) Bainbridge, F. A., S. H. Collins and J. A. Menzies. Proc. Roy. Soc. B **86**: 355, 1913.
(4) Barcroft, J. and T. G. Brodie. J. Physiol. **32**: 18, 1905.
(5) Beck, L. V. and R. Chambers. J. Cell. and Comp. Physiol. **6**: 441, 1935.
(6) Bieter, R. N. J. Pharmacol. and Exper. Therap. **43**: 407, 1931.
(7) Bieter, R. N. Am. J. Physiol. **97**: 66, 1931.
(8) Bieter, R. N. Proc. Soc. Exper. Biol. and Med. **30**: 981, 1933.
(9) Bieter, R. N. J. Pharmacol. and Exper. Therap. **49**: 250, 1933.
(10) Bieter, R. N. J. Pharmacol. and Exper. Therap. **53**: 347, 1935.
(11) Bordley, J., 3rd and A. N. Richards. J. Biol. Chem. **101**: 193, 1933.
(12) Cameron, G. and R. Chambers. Am' J. Physiol. **123**: 482, 1938.
(13) Chambers, R. Proc. Soc. Exper. Biol. and Med. **32**: 1199, 1935.
(14) Chambers, R. and G. Cameron. J. Cell. and Comp. Physiol. **2**: 99, 1932.
(15) Chambers, R. and R. T. Kempton. J. Cell. and Comp. Physiol. **3**: 131, 1933.
(16) Chambers, R., L. V. Beck and M. Belkin. J. Cell. and Comp. Physiol. **6**: 425, 1935.
(17) Chambers, R. and R. T. Kempton. J. Cell. and Comp. Physiol. **10**: 199, 1937.
(18) Chasis, H. and H. W. Smith. J. Clin. Invest. **17**: 347, 1938.
(19) Chasis, H., H. A. Ranges, W. Goldring and H. W. Smith. J. Clin. Invest. **17**: 683, 1938.
(20) Cullis, W. C. J. Physiol. **34**: 250, 1906.

(21) Defrise, A. Anat. Record 54: 185, 1932.
(22) De Haan, J. J. Physiol. 56: 444, 1922.
(23) Edwards, J. G. and L. Condorelli. Am. J. Physiol. 86: 383, 1928.
(24) Ellinger, A. Handbook der normalen und pathologischen Physiologie. IV. Resorption und Exkretion. Julius Springer, Berlin, 1929.
(25) Elsom, K. A., P. A. Bott and E. H. Shiels. Am. J. Physiol. 115: 548, 1936.
(26) Elsom, K. A., P. A. Bott and A. M. Walker. Am. J. Physiol. 118: 739, 1937.
(27) Forster, R. P. Proc. Soc. Exper. Biol. and Med. 38: 258, 1938.
(28) Gersh, I. and E. J. Stieglitz. Anat. Record 58: 349, 1934.
(29) Gibbs, O. S. Am. J. Physiol. 88: 87, 1929.
(30) Goldring, W., R. W. Clarke and H. W. Smith. J. Clin. Invest. 15: 221, 1936.
(31) Grafflin, A. L. Johns Hopkins Hosp. Bull. 48: 269, 1931.
(32) Grafflin, A. L. Biol. Bull. 71: 360, 1936.
(33) Grollman, A. J. Biol. Chem. 64: 141, 1925.
(34) Grollman, A. J. Gen. Physiol. 9: 813, 1926.
(35) Grollman, A. The cardiac output in health and disease. Charles C. Thomas, Baltimore, Md., 1932.
(36) Hayman, J. M., Jr. Am. J. Physiol. 72: 184, 1925.
(37) Hayman, J. M., Jr. Am. J. Physiol. 79: 389, 1927.
(38) Hayman, J. M. and A. N. Richards. Am. J. Physiol. 79: 149, 1926.
(39) Heidenhain, R. Pflüger's Arch. 9: 1, 1874.
(40) Hitchcock, D. I. J. Am. Chem. Soc. 48: 2870, 1926.
(41) Hoeber, R. Pflüger's Arch. 224: 72, 1930.
(42) Hoeber, R. J. Cell. and Comp. Physiol. 6: 117, 1935.
(43) Jolliffe, N. Proc. Soc. Exper. Biol. and Med. 28: 5, 1930.
(44) Kaplan, B. I. and H. W. Smith. Am. J. Physiol. 113: 354, 1935.
(45) Kempton, R. T. Am. J. Physiol. 119: 175, 1937.
(46) Kempton, R. T., P. A. Bott and A. N. Richards. Am. J. Anat. 61: 505, 1937.
(47) Landis, E. M., K. A. Elsom, P. A. Bott and E. H. Shiels. J. Clin. Invest. 15: 397, 1936.
(48) Marshall, E. K., Jr. Physiol. Rev. 6: 440, 1926.
(49) Marshall, E. K., Jr. Am. J. Physiol. 94: 1, 1930.
(50) Marshall, E. K., Jr. Am. J. Physiol. 99: 77, 1931.
(51) Marshall, E. K., Jr. J. Cell. and Comp. Physiol. 2: 349, 1932.
(52) Marshall, E. K., Jr. Proc. Soc. Exper. Biol. and Med. 29: 971, 1932.
(53) Marshall, E. K., Jr. Physiol. Rev. 14: 133, 1934.
(54) Marshall, E. K., Jr. and M. M. Crane. Am. J. Physiol. 70: 465, 1924.
(55) Marshall, E. K., Jr. and A. L. Grafflin. Johns Hopkins Hosp. Bull. 43: 205, 1928.
(56) Marshall, E. K., Jr. and A. L. Grafflin. J. Cell. and Comp. Physiol. 1: 161, 1932.
(57) Marshall, E. K., Jr. and A. L. Grafflin. Proc. Soc. Exper. Biol. and Med. 31: 44, 1933.
(58) Marshall, E. K., Jr. and H. W. Smith. Biol. Bull. 59: 135, 1930.

(59) Marshall, E. K., Jr. and J. L. Vickers. Johns Hopkins Hosp. Bull. **34:** 1, 1923.
(60) Mayrs, E. B. J. Physiol. **58:** 276, 1924.
(61) Nussbaum, M. Pflüger's Arch. **17:** 580, 1879.
(62) Pitts, R. F. Am. J. Physiol. **109:** 532, 1934.
(63) Pitts, R. F. Ann. Rept. Tortugas Lab., Carnegie Inst. of Wash. **36:** 90, 1936.
(64) Pitts, R. F. J. Cell. and Comp. Physiol. **11:** 99, 1938.
(65) Pitts, R. F. and I. M. Korr. J. Cell. and Comp. Physiol. **11:** 117, 1938.
(66) Ralli, E. P., G. J. Friedman and S. H. Rubin. J. Clin. Invest. **17**, 765, 1938.
(67) Richards, A. N. Am. J. Med. Sc. **170:** 781, 1925.
(68) Richards, A. N. and J. B. Barnwell. Proc. Roy. Soc. B. **102:** 72, 1927.
(69) Richards, A. N. and A. M. Walker. Am. J. Physiol. **79:** 419, 1927.
(70) Richards, A. N. and A. M. Walker. J. Biol. Chem. **87:** 479, 1930.
(71) Richards, A. N. and A. M. Walker. Am. J. Med. Sc. **190:** 727, 1935.
(72) Richards, A. N., P. A. Bott and B. B. Westfall. Am. J. Physiol. **123:** 281, 1938.
(73) Richards, A. N., B. B. Westfall and P. A. Bott. Proc. Soc. Exper. Biol. and Med. **32:** 73, 1934.
(74) Richards, A. N., B. B. Westfall and P. A. Bott. J. Biol. Chem. **116:** 749, 1936.
(75) Schmidt, C. F. and A. M. Walker. Proc. Soc. Exper. Biol. and Med. **33:** 346, 1935.
(76) Shannon, J. A. J. Cell. and Comp. Physiol. **4:** 211, 1934.
(77) Shannon, J. A. J. Cell. and Comp. Physiol. **5:** 301, 1934.
(78) Shannon, J. A. Am. J. Physiol. **112:** 405, 1935.
(79) Shannon, J. A. Am. J. Physiol. **113:** 602, 1935.
(80) Shannon, J. A. J. Clin Invest. **14:** 403, 1935.
(81) Shannon, J. A. Am. J. Physiol. **114:** 362, 1936.
(82) Shannon, J. A. Am. J. Physiol. **117:** 206, 1936.
(83) Shannon, J. A. Proc. Soc. Exper. Biol. and Med. **37:** 379, 1937.
(84) Shannon, J. A. J. Cell. and Comp. Physiol. **11:** 123, 1938.
(85) Shannon, J. A. J. Cell. and Comp. Physiol. **11:** 135, 1938.
(86) Shannon, J. A. Proc. Soc. Exper. Biol. and Med. **38:** 245, 1938.
(87) Shannon, J. A. J. Cell. and Comp. Physiol. **11:** 315, 1938.
(88) Shannon, J. A. Am. J. Physiol. **122:** 775, 1938.
(89) Shannon, J. A. Am. J. Physiol. **122:** 782, 1938.
(90) Shannon, J. A. Reanalysis of data contained in 76 and 77.
(91) Shannon, J. A. and S. Fisher. Am. J. Physiol. **122:** 765, 1938.
(92) Shannon, J. A. and H. Ranges. 1938. In preparation.
(93) Shannon, J. A. and H. W. Smith. J. Clin. Invest. **14:** 393, 1935.
(94) Sheehan, H. L. J. Physiol. **87:** 237, 1936.
(95) Smith, H. W. Quart. Rev. Biol. **7:** 1, 1932.
(96) Smith, H. W. The kidney in health and disease. Berglund and Medes, Lea & Febiger, Philadelphia, 1935.
(97) Smith, H. W. Biol. Rev. **11:** 49, 1936.
(98) Smith, H. W. J. Cell. and Comp. Physiol. **7:** 465, 1936.

(99) SMITH, H. W. The physiology of the kidney. Oxford University Press, N. Y., 1937.
(100) SMITH, H. W. AND R. W. CLARKE. Am. J. Physiol. **122**: 132, 1937.
(101) SMITH, H. W., W. GOLDRING AND H. CHASIS. J. Clin. Invest. **17**: 263, 1938.
(102) SMITH, W. W. AND H. A. RANGES. Am. J. Physiol. **123**: 720, 1938.
(103) VAN SLYKE, D. D., A. HILLER AND B. F. MILLER. Am. J. Physiol. **113**: 611, 1935.
(104) WALKER, A. M. AND K. A. ELSOM. J. Biol. Chem. **91**: 593, 1931.
(105) WALKER, A. M. AND C. L. HUDSON. Am. J. Physiol. **118**: 153, 1937.
(106) WEARN, J. T. Am. J. Physiol. **59**: 490, 1922.

PHYSIOLOGY OF HUMAN HAIR

C. H. DANFORTH

Department of Anatomy, Stanford University

Human hair is morphologically closely comparable to that of other mammals, but despite rather extraordinary regional specializations the range of hair forms is limited. For example, erectile tactile hairs, under voluntary control and of much physiological interest (Szymonowicz, 1937) are entirely lacking in man alone of all the mammals. Moreover, the differentiation of the pellage into long, stiff guard hairs and shorter dense under hair or wool, common especially in other than tropical forms, is at best only barely indicated in the human species. On the other hand there are few animals that show such sharp and abrupt contrasts in development of the hair on contiguous regions of the body, a fact which in itself suggests interesting problems in morphogenesis and the control of function. The general characteristics and conventional designations of different types of human hair may be found in textbooks of anatomy and in smaller special treatises (e.g., Danforth, 1925).

Interest in the hair may be directed toward its regional differentiations and morphology, toward the chemical and physical properties of the shaft, or toward the activity of follicles themselves. In this brief survey these aspects of the subject will be considered in this order, although it is of course apparent that all phases of the subject are mutually inter-related.

Sexual and individual differences in regional development of hair. As in a number of other mammals and many birds, whose feathers may be regarded as partially homologous with hairs, the cutaneous appendages of man show certain differences in their development in the two sexes. For the student of secondary sexual characteristics, hairs of the human body may be grouped roughly into three categories: 1, those that are essentially the same in all individuals, and apparently uninfluenced by sex hormones; 2, those that are similar in both sexes, but dependent on hormones which, so far as the hair is concerned, are apparently equivalent in the male and female; 3, those in which full development is normally limited to one sex. Not all the hairs of the body can be quickly assigned to one or another of these categories—many are

intermediate—but nevertheless such a grouping serves to bring to the fore certain points that are of considerable interest.

1. Hair that is the same in both sexes. Even before birth considerable regional differentiation has taken place in the pilary system, but during childhood the hair of the two sexes appears to be similar. During adolescence, maturity, and on into the later decades of life some of the successive hairs which appear in various regions undergo progressive change (Trotter, 1922), while other hairs in the same regions show, so far as has been ascertained, no change in type from childhood to old age. The latter, exemplified by the hair of the forehead, that of the infra-orbital region and indeed by the vellus[1] of the whole body, may be classed as hair uninfluenced by sex. Aside from the vellus there is probably other hair which is little if at all affected by sex hormones, but for the most part the evidence is not wholly satisfactory. To establish full status in this category no differences should be found in hairs from male, female, hypogonadic and castrate individuals.

2. Hair that behaves as an ambosexual character. The term ambosexual has been employed by Champy (1935) to designate those traits, especially common in birds, which require a gonadal hormone for their full manifestation but which can be activated by hormones of either the male or the female. Such traits are absent or underdeveloped in castrate individuals, present and similar, but not necessarily identical, in normal males and females. The hair of the axillary and pubic regions falls within this definition (Tandler and Grosz, 1910) and may therefore be characterized as an ambosexual feature. Much of the other terminal hair which tends to be present in both sexes may probably be appropriately assigned to this category, but the mere existence of a difference between castrate and normal individuals of the same and opposite sex need not in itself be adequate proof that a trait is of an ambosexual nature.

3. Hair that serves as a true secondary sexual character. The beard is a typical secondary sexual trait since it is normally present in men, absent in women and deficient or absent in individuals who have been agonadic from an early age. This does not necessarily imply that its

[1] For the fine hair of the human body which seems to be roughly homologous with wool, fur or under hair of various mammals, and which is commonly designated as wool, down, "lanugo" or fuzz, the writer some years ago introduced the term *vellus*. Despite its original meaning (Latin, fleece) it was hoped that this word might carry a definite connotation in the field of trichology. The term, which has not come into general use, is being given one more chance.

growth is conditioned directly by testicular secretion. Less clearly in this same category is the terminal hair of the chest, abdomen and shoulders. The size of individual hairs from the head may also be a secondary sexual character since Bernstein and Robinson (1927) believe that for a unit of length the weight averages greater in the male by some 18 per cent. Morphologically the difference between ambosexual and secondary sexual traits is obviously only one of degree.

4. Discussion of hair development in relation to sex. Until quite recently there has been a strong tendency to ascribe differences between the sexes to the influence of endocrine factor. Results of the earlier studies with ovarian hormones are largely responsible for this attitude, which is critically considered by Domm (1939). But the more recent results, even with birds, make it appear that the constitution of the crucial cells is, if anything, more important than the hormones which these cells may need, and often utilize, in realizing their potentialities. Among birds sexual differences in plumage are in some species wholly independent of sex hormones, in other species somewhat influenced by them, and in still others almost entirely dependent on one or more hormonal factors for their expression. The recent literature in this field is briefly reviewed by Danforth (1939). It is rather more difficult to get unequivocal evidence on the factors regulating the growth of hair. Nevertheless, it is clear that by proper selection hair follicles can be found showing an almost complete range of hormonal dependence and independence. That is, if the entire humoral complex of the male is considered as one factor and that of the female as another, there is good evidence that some hair follicles are uninfluenced by the difference between these factors, that the output of other follicles is affected to a slight, and that of still others to a marked degree by this differential. In each case it is apparently the constitution of the individual follicle which determines whether or not the humoral resources available to all the follicles will be utilized by this particular one in the development of a sex difference.

With respect to this aspect of the question we are confronted with two sets of problems. The one is to determine the constitutional peculiarities of the different classes of follicles and, if possible, how they are evolved in each individual, and the other is to determine the humoral components which individual follicles utilize or to which they are responsively "keyed." Unfortunately, satisfactory information in both of these directions is meager. As indicated above, certain individual hairs, or their follicles, fall into recognized categories and

behave in regular fashion. But two neighboring follicles may behave quite differently while those at some distance frequently show considererable conformity. It may be surmised that the reason why two apparently similar follicles both produce vellus hairs for eighteen or twenty years whereupon one begins to produce terminal hair while the other continues producing vellus to the end, or why two neighboring follicles produce normal supercilia for thirty or more years after which the product of one only becomes changed, is in some way dependent on differential qualities imparted to the respective follicles at the time of their organization. So far as the writer is aware there is no direct evidence in support of this view, but there is ample evidence that something of the sort is true of feather follicles, and one cannot follow over a period of years the small, often microscopic, irregularities and blemishes in an area of human skin without being impressed by the high degree of autonomy shown by groups of epithelial cells. Finally, it is important to recognize that there are marked hereditary and racial differences between homologous follicles of different individuals.

Analysis of humoral factors in hair development is in a scarcely more satisfactory state than that of the constitutional factors. There is an immense amount of clinical data which seems to be more or less significant in this connection, but in the nature of the case such data are usually not well controlled. One item stands out rather above the others, an impressive association between tumors of the suprarenal cortex in children and precocious and extensive development of the pilary system, especially its ambosexual and secondary sexual components. The picture is usually complicated by a general sexual precocity which leaves the relations somewhat obscure. It has been suggested that the, possibly abnormal, secretion of the affected cortex directly stimulates the hair; that the suprarenal effect is upon the gonads which in turn stimulate the hair; and that some more complicated hormonal imbalance is responsible for the observed phenomenon. Next to the suprarenals (at least the diseased suprarenals) the testes, and then perhaps the thyroid and ovaries, show evidence of a more or less direct relation to hair growth in some specialized areas, but it is probably correct to say that we really have no satisfactory information on the relation of any of the endocrines to hair growth in man. The literature in this field is indeed extensive, and many of the clinical contributions contain data that undoubtedly will be of much value in a final analysis of the rôle of endocrines in hair production, but to attempt such an analysis at this time would seem to be definitely premature.

In the light of such knowledge as we have, it cannot be emphasized too strongly that the pilary system taken *as a whole* is ill adapted for an analysis of specific hormonal effects and that, even when the tests are limited to a particular type of hair, marked individual differences are to be expected. For example in eunuchs castrated in early childhood, hair of an ambosexual character may be entirely lacking or show a sparse or even moderate development (Wagenseil, 1927). Those follicles which produce hair which may properly be considered as of a secondary sexual character apparently are, as might be expected, most sensitive to different levels of gonadal, especially testicular, hormones while ambosexual hair, although responding quickly to the presence of appropriate hormones, is possibly somewhat less sensitive to their absence. These inferences are drawn from the earlier observations on eunuchs and on scattered and fragmentary data in clinical and experimental papers appearing during the last few years, especially since the introduction of testosterone, androsterone and their variants.

Whether or not there is a true hormonal antagonism with reference to any part of the pilary system is an open question. Certainly the evidence in favor of it is not extensive, but the old idea that testicular and ovarian secretions have opposite effects on the capillus seems to have some support despite the fact that baldness and probably "thinning" of the hair in males is due in part to a sex-linked gene (Osborn, 1916; Snyder and Yingling, 1935).

The autonomy of the skin and hair follicles in different regions of the human body is apparently of the same order as the autonomy of skin and feather follicles in birds and, by inadvertence, may sometimes be tested in the same way. Occasional cases occur in which full-thickness autoplastic skin grafts made in childhood ultimately develop, in an entirely new site, the type of hair appropriate to the region from which the graft was taken. In birds it is indicated by experiment, and in man by inferences, that strictly comparable follicles (e.g., those of facial hairs of Semitic and Mongolian subjects), may or may not respond to a given humoral complex depending entirely upon the genotypes involved. In other words, different parts of the same body and the same parts of different bodies diverge from each other in much the same way. That these racial differences in response are primarily constitutional, having arisen by differentiation during development, now seems much more probable than that they are due to different endocrine levels as was formerly suggested by Sir Arthur Keith. For any given individual it seems likely that all of the follicles have about equal chances at all the

hormones in the circulation, and that the contribution of the hormone to follicular function is in each case determined by some kind of selective action on the part of the follicle itself.

PROPERTIES OF THE HAIR SHAFT. In most members of most races the product of a typical hair follicle is an elongated slender ribbon which might be compared roughly to an attenuated scale having an upper and lower surface, a medial and lateral side. The "hair-slopes" are such, however, that the sides of some of the hair shafts may fall in almost any plane. Divergence in the direction of contiguous hair slopes gives rise to "natural parting" of the hair or to "crests" or whorls (Kidd, 1903), such for example as are commonly seen at the vertex, on the side of the head, on the forehead and the median part of the (usually left) eyebrow (Bolk, 1924; Upham and Landauer, 1935). It is interesting that the arrangement and direction of the whorls appear to be inherited in regular Mendelian fashion (Schwarzburg, 1927), which sets another problem in the physiology of development.

1. Curling and twisting of the shaft. While most hairs are flattened to a degree not often suspected by those unaccustomed to examining them in a rotator, there are some terminal hairs which are practically circular in cross section so that the index $\left(\frac{\text{lesser diameter} \times 100}{\text{greater diameter}}\right)$ approaches or even equals 100. For example, in samples of capillus hair from Indians or Chinese, the range in index often runs from somewhere in the 60's to approximately 100 (Woodbury and Woodbury, 1932). Whether the hair index ever exceeds 100, i.e., whether some hairs are thicker than they are wide, would be difficult to determine.

It is probably just to say that after 85 years (since Peter Browne, 1853) of insistence on the anthropological importance of the cross-sectional form of hair, we are still far from a satisfactory knowledge of the subject. It is commonly stated that hair with a circular cross section is straight while that with a cross section which is oval in outline is curly. Indeed a recent study of Indian hair (the Woodburys') classifies a number of tribes into curly, wavy, and straight haired groups, not apparently on the actual appearance of the hair but on its index; 50–75 being curly, 75–80 wavy, and 80–100 straight. But there would seem to be no reason why a shaft that is uniform in structure should curl merely because its cross section is elliptical in outline; and it frequently does not. If, however, the upper and lower sides of the shaft were of different density, or if the transverse axis did not bisect the vertical, a differential would be provided which might be expected to

readily induce curling. While many curly hairs clearly do show a kidney shaped cross-sectional outline, or one in which the shorter axis of an imperfect ellipse is not bisected by the longer, there are others in which any eccentricity in the form of the cross section is not apparent, and in such hairs it is possible that the differential is purely in the density of cornification. That hairs with very diverse cross-sectional form may or may not curl is shown by the studies of Kneberg (1935).

Straight hairs can readily be made to curl, at least temporarily, by drawing them between the nail of the index finger and ball of the thumb in such a manner as to compress one side more than the other. If in performing this experiment the pressure is successfully graduated, the artificially deformed hair will often coil in the form of a watch spring. Done under proper conditions of heat and moisture, the deformation becomes fixed, a fact which is utilized in producing a "permanent wave." When the pressure exerted is constant over a considerable stretch of shaft, the curvature tends to have a constant radius so that the hair is forced to coil in the form of a spiral. This is the usual form assumed by curly or wavy hair, the particular grade depending on the radius of curvature. An occasional hair will naturally take the watch spring form if moistened and allowed to dry in the absence of external deforming agents. Generally, however, various factors, such as combing, tend not only to draw the coil out like a corkscrew, but also to twist the hair on its own axis so that the shaft comes to spiral alternately to the right and the left. Kettler (1932) is of the opinion that while curling may be an intrinsic property of the hair shaft, the twisting of the shaft on its own axis is purely fortuitous. The latter contention is probably correct for a certain number of cases, but there still remains the probability that there is also an inherited tendency to twisting. This would seem the more probable since there are racial differences in the form of this twisting. The frequency of twists in the shaft is an important factor in determining the quality of the hair as observed en masse. When such twisting of the shaft occurs in head hair it tends to be more or less uniform with a very gradual rotation of the axis, but in pubic and axillary hair the turns are abrupt, giving an effect of crispness. The causation of this twisting of the shaft may still be regarded as an open question. That a natural curl or wave in the hair is genetically determined has been fairly well established. Kettler (1932) from the study of 100 families was led to conclude that among Europeans curly, wavy, straight correspond to the three possible combinations of a single pair of genes, wavy representing the heterozygous condition.

2. Pigmentation. Extensive work on the chemistry and morphology of hair pigments in laboratory and domestic mammals (e.g., Bloch, 1927) has unfortunately not been paralleled in studies on human hair (cf. Conitzer, 1931). In man as in many other forms both diffuse and granular pigments occur. The former apparently account for all tones of red, while the latter give the various shades of brown which at their greatest intensity pass for black. On genetic grounds there is evidence that glossy black hair carries both diffuse and granular pigment which may be inherited separately, but the genesis and cytology of these pigments have not been adequately studied. Interesting preliminary observations on the distribution and grouping of pigment granules in human hair were reported by Hausman (1925). Albinism in man seems to affect the granular more than the diffuse pigments. Dominant white spotting (Holmes and Schofield, 1917) is well known in man, but whether it is due to the local production of an antioxidase as claimed by Onslow (1917) for the rabbit is unknown. The degree to which light and heat influence the genesis of pigment in the hair follicle does not seem to have been adequately studied. Bleaching of the hair under the influence of light is a familiar phenomenon. Hairs of the head often show gradations between tip and base, and the age of individual hairs on the back of the hand, where the cycles are short, can often be estimated by the inverse relation between age and intensity of pigmentation. Pigmentation, like other characteristics, shows considerable regional specialization.

3. Chemistry of hair. It does not seem expedient at this time to review work on the chemical composition of human hair. Various analyses (e.g., those of Rutherford and Hawk, 1907; E. Voit, 1930) show a fair constancy in the proportions of different elements, but with variations up to possibly 5 per cent of the mean. These deviations have been thought to be associated with age, race and sex, but at times they seem to be as great within the groups as between them. In hair from barber shops, Vickey and Leavenworth (1929) obtained approximately the following figures in per cent for some of the more important amino-acids: arginin, 80; lysine, 2.5; histidine, 0.5. Cystin represented about 16.5 per cent of all amino-acids present. Analyses of hair of animals under varying conditions of health and nutrition are rather extensive.

It is frequently stated that the hair serves as an important organ of excretion. Lead in particular has been supposed to be one of the substances that is eliminated through the hair in appreciable amounts, but Melnick and Cowgill (1938) from a combination of chemical analysis

of human hair and experiments with animals came to the conclusion that no excretion of this sort occurs, the apparent increased lead content of hair from painters and others being due to contamination with lead dust. Since lead that gets into the hair from the air is very difficult to remove, except chemically, these authors suggest routine examination of hair samples from workers exposed to lead dust as a reliable means of determining the degree of hazard to which they are subjected. It remains to be seen if the supposed excretion of antimony and arsenic will be similarly explained.

One of the interesting, and probably very significant, developments of the last few years is due chiefly to biochemists in the textile industries who have attacked the problem of determining the molecular structure of protein fibers. Human hair has been used to some extent in these studies. On the basis of x-ray examinations it is believed that the keratin fiber consists of a series of zigzag (polypeptide) chains connected by side chains, an arrangement which provides for the type of deformation referred to in section 1 above and also accounts both for the normal elasticity of hair and for the possibility of a "set." When deformation occurs in the presence of steam, a reorganization is effected with a new spacing of the side chains. A few of the papers in this field by Astbury and his co-workers are listed in the bibliography.

4. Physical properties of the hair shaft. Tensile strength of the hair shaft is more a matter of physical than physiological interest. The pull required to break a hair is almost always greater than that required to pull it from its follicle. Basler (1925, 1927) and after him others have investigated this matter. Basler's technique consisted essentially in suspending a small pan from the end of a hair in such a way that the pull was directed along the axis of its follicle. Then 3-gram weights were added until the hair was pulled out. In this laboratory Mr. Eugene Cronkite replaced the pan by a paper cup of known weight into which water from a graduated burette could be run until the hair was released from its follicle. This method permits a considerable degree of accuracy in the determinations.

Individual hairs differ considerably in their resistance to pull. For hairs of the scalp in seven Europeans, Basler obtained values ranging from 25.4 to 36.9 grams, with an average of 31.89 grams. For seven Chinese and Japanese the range was from 32 to 53 grams, with an average of 48.4 grams. In this laboratory a number of white subjects showed individual ranges as great as from 12 to 43 grams but the averages found by Mr. Cronkite are not very different from those of Basler. The firm-

ness of attachment is not the same in all parts of the scalp. As pointed out by Basler, if a single head hair will support on the average about 31 grams, it would take some 2100 of them to support 150 pounds or the weight of a medium sized man. This is perhaps 20 per cent of the hairs on the scalp.

In reporting some of the data, the morphology of the hair root and bulb seems to have been ignored. When a hair is actively growing the proximal part is soft, and on being extracted stretches into a long tapering thread. A hair in the early part of the resting stage has a dilated cornified bulb which does not stretch and which offers considerable resistance to pull. Toward the end of the resting stage the bulb is freed at the base and slowly moves toward the surface offering progressively less resistance. With a little magnification these points may be demonstrated on the back of the hand where new hairs can be identified by their darker (less bleached) coloring. It is noticeably less painful to pull out a growing hair than one that has just completed its growth.

One other aspect of the mechanics of the hair shaft relates more properly to the arrector pili muscle which, running from the skin to the lower side of the follicle, tends on contraction to straighten or erect the hair. In a study of cutis anserina Beck (1938) employed an ingeniously devised apparatus in which the arm of each subject rested in a specially prepared cast and the one remaining hair in an area otherwise shaved clean was definitely centered with reference to an adjustable wire. To this setup was added a time marker which changed its position at 0.5 second intervals. A camera and a suitable source of light was then brought into alignment with the hair and the surrounding area rapidly cooled. Films run through the camera showed the hair to rise somewhat from its more or less horizontal position, due to contraction of the muscle on cooling. The time required for maximum erection was found to be somewhat variable, apparently from 6 to 12 seconds. The degree of erection of the shaft as measured by the angle made with the surface of the skin varied from 3° to 9.5°. Beck calculated that in producing this grade of "goose flesh" (the maximum ?), the arrector pili muscle shortens by about $\frac{1}{4}$ of its resting length. The contractions show several maxima, or phases, and seem to be tetanic in character.

Cyclic activity of hair follicles. *1. The normal cycle.* In the production and replacement of hair each follicle passes through a succession of active and quiescent periods, for which the hair shaft itself furnishes a convenient index. Every follicle apparently has its own cycle which in terms of percentage deviations from the mean compares

favorably with other rhythmic functions. In regions where follicular activity has been studied most carefully the cycles are found to be highly individual, often differing markedly in follicles separated by only a fraction of a millimeter.

Accurate and detailed information on the characteristics of the cycle is not extensive. Trotter (1924) has determined lengths of the active and quiescent phases in follicles from a number of regions. Pinkus (1924), Bulliard (1923) and some others have also made a limited number of fairly definite determinations, while not a few, including Friedenthal (1908), have attempted more or less plausible estimates of the duration of the cycle in various types of hair. For example, since the capillus hair grows about 0.4 mm. a day and normally reaches a length of some 65 cm., it has been inferred that there is a period of continuous growth lasting about 1600 days (Stöhr, 1903). It is probably rarely longer than this. The implication of Griffith (*Silver threads among the gold*, 1933) that if it were not for attrition and barbers the hair of elderly people would trail behind them for as much as 35 feet need not be considered as other than the pleasantry which was no doubt intended. The duration of the resting phase in follicles of the scalp has not been satisfactorily determined, but it is believed to be short.

In a general way it appears that follicles that produce the larger and longer hairs, show a greater output in a unit of time and are active more continuously than follicles which produce shorter hairs. In a series of pregnant and post-parturient women Trotter (1935) found that follicles of the small hairs of the lumbar region had a short growth and long rest period, those of the relatively large pubic hairs a growth and rest period of approximately equal length, and the still larger perineal hairs a growth period slightly in excess of the resting period. A similar relation between active and quiescent periods is also noticeable among larger and smaller hairs that occur close together within the same region.

2. Normal fluctuations in the cycle. The literature on the subject generally implies that the rate of production is uniform for each follicle during the growth period, and measurements taken at intervals and plotted against a time scale usually do fall very nearly in a straight line. However, frequent and careful measurements show that, at least in some types of hair, there is a short period of rather smooth acceleration at the beginning of growth and a longer period of somewhat irregular retardation at the end. A sample of hairs from the index finger showed a lower average rate for the first ten days of their growth than for the second. Any such difference in rate is not likely to appear when meas-

urements are taken only once or twice a week. Moreover, it is possible that there are minor fluctuations occurring irregularly and at short intervals throughout the entire period.

Verification of the latter type of fluctuation, if it exists, is not easy because of the difficulty in measuring the exposed shaft of a hair accurately to within a few hundredths of a millimeter. The measurements, of course, have to be made under sufficient magnification, and this tends to give some optical aberration when a large field is observed, as is often the case when the hair is placed flat against the skin under a transparent rule or held alongside a scale in the vertical position.

A further difficulty is presented by the ease with which the portion of the shaft beyond the level of the skin can be increased by slight tension, or, conversely, the skin at the mouth of the follicle depressed by light pressure. The present writer prefers a finely graduated metal rule with one end filed on the back to a thin edge. It is desirable to have the width of the rule sufficient so that any slight pressure will be distributed over a number of millimeters or even a centimeter of skin. This sharp edged rule is brought up to the under surface of the hair as it emerges from the skin and tilted slightly so that the shaft lies flat against the graduations, care being exercised to have the pressure exerted at each measurement as slight and as constant as possible. The accuracy of this method as determined by repeated measurements of the same hairs is still not so great as might be desired, but usually falls well within 0.1 mm. with occasionally a long series of measurements in which there are no deviations.

Several factors other than uneven growth might be thought of as possibly contributing to an appearance of irregularity. Variations in the turgidity of the tissues is apparently not important. On one occasion just after a number of hairs on the finger had been measured the subject became the victim of a bee sting which resulted in a sudden rise in pulse rate and an appreciable transient edema of the extremities. Measurements made before, during and after the edema checked very closely. Application of heat and cold to the skin showed a similar lack of influence on the length of the exposed part of the hair. Apparently the follicles generally rise and fall with the skin in which they are embedded, without much change in the relative amounts of shaft above and below the surface.

A frequent source of possible error is the loss of the end of a hair shaft between measurements. While appreciable wear on the shaft is not common except where there is direct and rather regular friction

(e.g., a sleeve or bandage playing over the inner malleolus at the wrist), the frequency with which the end of a shaft is cut, burned or broken off is rather surprising. The occurrence of some of these accidents, especially burns, can easily be detected by examining the end of the shaft but at times it is difficult, if not impossible, to tell whether a sudden apparent retardation is due to an actual slowing or merely to a loss of the end of the shaft since the preceding measurement. But bearing in mind all these possible disturbing factors, there still seems to be ground for suspecting a small degree of irregular fluctuation during the entire growth period. The following increments in tenths of millimeters taken at approximately 24 hour intervals from a hair on the index finger show a fairly typical set of figures: 1, 2, 5, 0, 6, 2, 3, 3, 4, 3, 3, –, –, (0.5 mm. in two days), 0, 4, –, 3, 5, 2, 3, 4, 2, –, –, (0.5 mm. in the two days), 1, –, –, (0.4 mm. in two days), 1, 4, 5, 0, 3, 0, 0, 3, 5, 3, 1, 3, 0.

Whether or not there is a diurnal fluctuation in the rate of hair growth has not been answered satisfactorily. Of 134 pairs of measurements of growth between 9 a.m. and 9 p.m. and between 9 p.m. and 9 a.m., the day and night growth were the same in 30 instances. In 36 cases the day period showed less growth than the night period, and in 68 cases the reverse was true. The sum of all the growth during the periods from 9 a.m. to 9 p.m. was 56 per cent of the total.

Seasonal variation in the growth of head hair has been studied to some extent by Voit (1930) whose observations are open to the criticism that they do not differentiate growth and rest periods, nor take any account of individual follicles. The total output of hair as measured by weighing, Voit finds to be greater from March to July than from August to February. In some personal observations on hairs of the hand the average length of cycles was found to be slightly greater from October to May than from May to October. These two sets of observations, both inadequate, are quite possibly consistent, but more precise information is needed.

Despite minor fluctuation, real or spurious, the picture of the cycle as a whole presents a considerable degree of consistent regularity. The same follicle over a period of years will frequently show many cycles of almost precisely the same length, although there are occasional deviations of up to 20 per cent of the mean. Unfortunately the literature on the subject presents few reports on individual follicles observed over more than two or three cycles, an exception being the case of a naevus hair on the chest which Pinkus (1924) observed more or less frequently for seven years. The writer has unpublished notes on 54 individually

identified follicles which he has studied from time to time during the past fifteen years. Figure 1 indicates the output of three such follicles during two different cycles separated in each case by a ten year interval. Although these three follicles are from a region on the back of the hand where the hair shafts are relatively uniform in size, there is a conspicuously closer agreement between curves for the same follicle

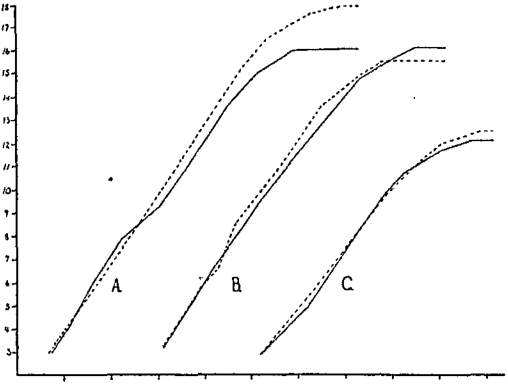


Fig. 1. Curves showing increase in length of the hairs from each of three follicles on the index finger of an adult man. In each of the pairs, *A*, *B*, and *C*, the solid line corresponds to a hair produced when the subject was at the age of forty and the broken line to a hair developed from the same follicle ten years later. The six hairs all grew at approximately the same season of the year. The scale at the left represents lengths in millimeters, the divisions at the bottom correspond to ten day periods.

functioning at different times than for different follicles functioning at the same time.

On the fingers, where the relative differences in hair size are usually greater than on the back of the hand, functional autonomy of the follicles is even more marked. Figure 2 shows the characteristic rhythmicities of eight follicles over a period of four years. Three follicles which are situated within a circle with a radius of less than 0.5 mm., have maintained their own rhythms during the fifteen years in which they have been under observation. The cycles of each of them have fluctuated

somewhat, but with no apparent tendency to deviate in the same direction at the same time. At any given moment one, two, or all three may be either active or quiescent. In this particular group the lengths of the respective cycles have been approximately 72, 78, and 125 days.

3. Attempts at artificial modification of the cycle. In figure 2, times when hair shafts were pulled from their follicles are indicated in several places. When the hair broke, even deep within the follicle, there was no obvious effect on the cycle. But generally, if not always, when the hair was completely removed early in the cycle, a short period of rest supervened followed by a new and usually normal cycle. No clear indication of a subsequent compensation in cycle length was detected,

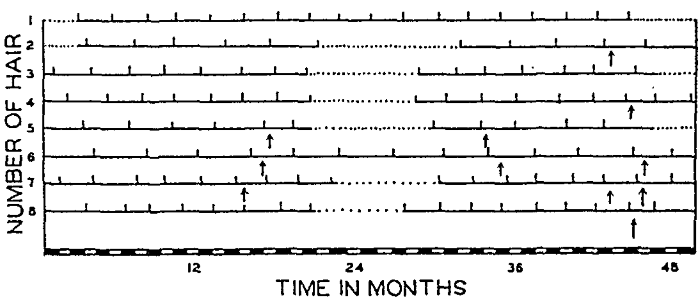


Fig. 2. Variations in lengths of cycles in eight follicles (numbered 1–8) located on the index finger and observed over a period of four years. The time scale at the bottom represents months, while the lines above are broken into segments corresponding to successive cycles in each of the eight follicles. Arrows indicate dates at which growing hairs were pulled from their follicles. This series of records began and ended in November. Dotted segments of the line correspond to periods during which observations were not recorded.

the interruption of the growth phase seeming, as it were, to establish a new base line.

Carefully conducted attempts to regulate or even modify the cycle by indirect means have met with little success. Trotter (1923) was unable to detect any stimulation from shaving the hair of different regions or from the persistent application of cold cream. The writer failed to find any effect on hair follicles of the fingers (where there are some differences associated with sex) following the vigorous application two or three times a day for a week of theelin, testosterone propionate, or cortical hormone. Hairs cut to facilitate measurements rarely show an increased growth during the following hours or day but in my own records frequently a slight decrease, which, however, is probably of no

statistical significance. The number of hair "restorers" or hair "stimulators" that have proved wholly ineffective is very great. Nevertheless despite the highly refractory character of the hair follicle, it is obviously not entirely immune to either internal or external influences. The factors that really can affect the function of the follicle have been studied more effectively in lower mammals than in man. In both, such fundamental influences as disease, abnormal nutritional states, vitamine and hormonal deficiencies frequently do show an unmistakable influence on the folliclular output, the exact degree and nature of the effect often being difficult to evaluate because of the considerable lag in response. There seems to be little doubt that in some way ultra violet radiation also has a mild effect on the hair follicle. Whether or not this accounts for the slight seasonal difference already referred to has not been determined. The superficially similar toxic effects of thalium on the follicles in man, perhaps colchicine in mammals and thyroxin in birds, serve for the present to suggest one possible approach to the study of the reactions of hair and feather follicles.

SUMMARY. Knowledge of the human pilary system is not so extensive as its potential importance would warrant. From a physiological point of view the functioning of individual hair follicles proves to be especially interesting. Follicles are found to show a remarkable degree of autonomy, which is maintained over long periods and is not readily disturbed by external factors. Each follicle has its own individual rhythm which is relatively constant and frequently does not synchronize with the rhythms of neighboring follicles. The general characteristics and potentialities of the follicle are possibly imparted to it during fetal development, at any rate the follicles of certain regions show specializations which suggest some form of developmental predetermination. To what extent peculiarities of a secondary sexual or ambosexual nature are conditioned by constitutional, and to what extent by hormonal or other humoral factors, is as yet very imperfectly understood. The follicles afford especially favorable material for the study of physiological rhythmicity and related problems.

REFERENCES

(More extensive bibliographies will be found in several of the articles listed below, especially those of Trotter, Landauer, Conitzer, Hausman and Danforth.)

ASTBURY, W. T. Fundamentals of fibre structure. Oxford University Press, 1933, p. 188.

X-ray studies of protein structure. Cold Spring Harbor Symposia on Quantitative Biology 2: 15, 1934.

Recent advances in the x-ray study of protein fibres. J. Text. Inst. 27: 281, 1936.

Astbury, W. T., H. J. Woods, J. B. Speakman, J. L. Stores and C. S. Whewell. The structure of keratin fibres. J. Text. Inst. 28: 394, 1937.

Basler, A. Über die Wurzelfestigkeit der Kopfhaare bei Chinesen. Ztschr. f. Morph u. Anthrop. 26: 247, 1927.

Beck, R. Über Gänsehaut. Inaugural-Dissertation, Breslau, 1938, 10 pp.

Bernstein, M. and S. Robertson. Racial and sexual differences in hair weight. Am. J. Phys. Anthrop. 10: 379, 1927.

Bloch, B. Das Pigment. Handbuch der Haut-und Geschlichtskrankheiten, Berlin, 1927.

Bolk, L. On the hair slope in the frontal region of man. J. Anat. 53: 208, 1924.

Bogart, R. and H. L. Ibsen. The relation of hair and skin pigmentation to color inheritance in cattle, with some notes on guinea-pig hair pigmentation. J. Genetics 35: 31, 1937.

Brown, P. A. Trichologia Mammalium; or a treatise on the organization, properties and uses of hair and wool; together with an essay upon the raising and breeding of sheep. Philadelphia, 1853.

Bulliard, H. Influence de la section et du rasage repétité sur l'evolution du poil. Ann. de Dermat. et Syph. 4: 386, 1923.

Champy, Ch. Recherches sur l'action des glandes génitales le plumage des oiseaux. Arch. Anat. Micr. 31: 145, 1935.

Conitzer, H. Die Rothaarigkeit. Ztschr. f. Morph. u. Anthropol. 29: 81, 1931.

Danforth, C. H. Hair with special reference to hypertrichosis. J. A. M. A. 1925, pp. 152.

Relation of genetic and endocrine factors in sex. Chapter 6 in Sex and internal secretions. 2nd ed., 1939 (in press).

Domm, L. V. Modification in sex and secondary sexual characters in birds. Chapter 5 in Sex and internal secretions. 2nd ed., 1939 (in press).

Friedenthal, H. Beiträge zur Naturgeschichte des Menschen. (Das Haarkleid des Menschen.) Jena, 1908.

Griffith, I. Silver threads among the gold, being the story of human hair. Am. J. Pharm. 105: 159, 1933.

Hausman, L. A. The relationships of microscopic structural characters of human head hair. Am. J. Phys. Anthrop. 8: 173, 1925.

A comparative study of the structural elements of human head hair. Am. Nat. 59: 529, 1925.

Holmes, S. J. and R. O. Schofield. Inheritance of white forelock. J. Heredity 8: 359, 1917.

Kettler, G. Die Krümmung des menschlichen Kopf-Haares und ihre Vererbung. Ztschr. f. Konstitutionslehre 16: 559, 1932.

Kidd, W. The direction of hair in animals and man. London, 1903, pp. 154.

Kneberg, M. Improved technique for hair examination. Am. J. Phys. Anthrop. 20: 51, 1935.

Hair weight as a racial criterion. Am. J. Phys. Anthrop. 21: 279, 1936.

Landauer, W. Die Vererbung von Haar- und Hautmerkmalen, ausschliesslich Färbung und Zeichnung, mit Berucksichtigung von Rassedifferenzierung und Deszendenz. Ztschr. f. induk. Abstammungs- u. Vererbungslehre 42: 113, 1926.

MELNICK, J. L. AND G. R. COWGILL. Lead analyses of hair as an indication of exposure to lead. Proc. Soc. Exper. Biol. and Med. 38: 899, 1938.
ONSLOW, H. A. Contribution to our knowledge of the chemistry of coat color in animals and of dominant and recessive whiteness. Proc. Roy. Soc., London, Series B. 89: 36, 1917.
OSBORN, D. Inheritance of baldness. J. Hered. 7: 347, 1916.
PINKUS, F. Zur Kenntnis der Lebensdauer der menschlichen Terminalhaare. Ztschr. f. Morph. u. Anthrop. 24: 265, 1924.
RUTHERFORD, T. A. AND P. B. HAWK. A study of the comparative chemical composition of the hair of different races. J. Biol. Chem. 3: 459, 1907.
SCHWARZBURG, W. Statistische Untersuchung über den menschlichen Scheitelwirbel und seine Vererbung. Ztschr. f. Morph. u. Anthrop. 26: 195, 1927.
SNYDER, L. H. AND H. C. YINGLING. Studies in human inheritance. XII. The application of the gene frequency method of analysis to sex-influenced factors, with especial reference to baldness. Human Biol. 7: 608, 1935.
STÖHR, P. Entwicklungsgeschichte des menschlichen Wollhaares. Anat. Hefte 23: 1, 1903.
SZYMONOWICZ, W. Vergleichende Untersuchungen über die Innervation der Sinushaare bei den Saugern II. Ztschr. f. Anat. u. Entwicklungsgesch. 106: 85, 1937.
TANDLER, J. AND S. GROSZ. Über den Einfluss der Kastration auf den Organismus III "Die Eunuchoide." Arch. Entwicklungsmech. d. Organism 29: 290, 1910.
TÄNZER, E. Die mechanischen Eigenschaften des menschlichen Kopfhaares. Pflüger's Arch. 208: 671, 1925.
TROTTER, M. A study of facial hair in the white and negro races. Washington Univ. Studies 9: 273, 1922.
The resistance of hair to certain supposed growth stimulants. Arch. Derm. and Syph. 7: 93, 1923.
The life cycles of hair in selected regions of the body. Am. J. Phys. Anthrop. 7: 427, 1924.
The hair. Section II, Special Cytology. Edmund V. Cowdry, editor, p. 39, 1932.
The activity of hair follicles with reference to pregnancy. Surg. Gyn. and Obst. 60: 1092, 1935.
UPHAM, E. AND W. LANDAUER. The relation of thickness of cutis and subcutis to hair slope in human skin. Anat. Rec. 61: 359, 1935.
VICKERY, H. B. AND C. S. LEAVENWORTH. The separation of cystin from histidine: the basic amino-acids of human hair. J. Biol. Chem. 83: 523, 1929.
VOIT, E. Ueber die Grösse der Erneuerung der Horngebilde beim Menschen. I. Mitteilung. Die Haare. Ztschr. f. Biol. 90: 508, 1930.
WAGENSEIL, F. Beiträge zur Kenntnis der Kastrationsfolgen und der Eunuchoidismus beim Mann. Ztschr. f. Morph u. Antrop. 26: 264, 1927.
WOODBURY, G. AND E. T. WOODBURY. Differences between certain of the North American Indian tribes as shown by a microscopical study of their head hair. State Museum, Denver, Colorado, 1932, p. 37.
WOODS, H. J. Fibre forms in animal hairs. Nature 136: 262, 1935.

RESPIRATION IN DIVING MAMMALS

LAURENCE IRVING

Edward Martin Biological Laboratory, Swarthmore College, Swarthmore, Pa.

The diving mammals offer to the physiologist a natural experiment which shows how long and by means of what respiratory adjustments a mammal can endure asphyxia. The respiratory adjustments which allow such endurance to the diver involve organs which in structure and operation are qualitatively like those of land animals; but in the divers the operation of the typical respiratory devices is quantitatively accentuated so that the animal as a whole can resist asphyxia. Periods of deliberate apnea lasting for ten minutes may be observed in the divers; respiratory cardiac arrhythmias may reduce the frequency of the heart by fifty per cent for several minutes; large local changes in peripheral blood flow and sometimes in arterial pressure indicate the extensive peripheral vascular changes which are the principal physiological adjustments for the endurance of asphyxia.

These conspicuous respiratory reactions of divers are qualitatively identical with the brief and inconspicuous adjustments by means of which mammals generally attempt physiological compensation for asphyxia. If we consider the quantitative development of some of the respiratory faculties of divers, we can make use of their accentuated physiological adjustments to clarify some of the obscure but crucial problems in mammalian physiology which are involved in the simultaneous control of breathing, cardiac action and the peripheral vascular bed during respiratory stress.

Men can hold their breath voluntarily for about 45 seconds without any preliminary deep breathing (Hill, 1912). Three hundred and eighteen American aviators examined by Schneider (1930) could hold their breath on the average for 68 seconds. Twenty-five men accustomed to work in diving equipment held their breath on the average for 91 seconds (Shilling, Hansen and Hawkins, 1934). The preparation was to expire deeply once and then inspire. The maximal endurance for any individual was two minutes and forty-seven seconds. These observations cover a group of men selected for physical ability and probably indicate the limits of human endurance of voluntary apnea.

Preliminary deep breathing allows for endurance of voluntary apnea up to five or six minutes, or in one case to nine minutes (Hill, 1912). A group of college students who on the average could hold their breath for 68 seconds without preparation, could hold out for from 65 seconds to 4 minutes and 20 seconds after 2 minutes of forced breathing (Schneider, 1930).

Inhalation of oxygen at least doubles the ability to maintain voluntary apnea (Hill, 1912; Schneider, 1930) after forced breathing. One individual held his breath for 15 minutes after forced breathing and inhalation of oxygen. When the Japanese swimmers in the Olympic games at Los Angeles inhaled oxygen before the races, they conspicuously excelled the other contestants (Robertson, 1935). These experiments show that depletion of the initial amount of carbon dioxide and increase in the store of oxygen separately or in combination can greatly extend the period of holding the breath. Neither form of preparation is possible for sudden submersion, whether it is made voluntarily or under compulsion.

The accumulation of carbon dioxide gradually induces disagreeable subjective symptoms, while acute deficiency of oxygen suddenly causes faintness without preliminary warning sensations if carbon dioxide does not accumulate. The interplay of the two conditions upon endurance of asphyxia is perceptible but not easy to evaluate separately. The concentration of carbon dioxide which may develop during voluntary apnea is unpleasant but not dangerous; the depletion of oxygen to a dangerous degree may scarcely be recognized by the victim. It appears that lack of oxygen is a factor against which the mammal has little sensory protection and scant reserves. On the other hand, the capacity of the body for carbon dioxide is so large that with any reasonable opportunity being given to distribute carbon dioxide among the buffers of the tissues, such as occurs with an active circulation during exercise, the carbon dioxide which accumulates in acute asphyxia is probably not the critical check upon the endurance of a determined individual.

Since the time of early records human divers have operated in sponge, pearl and shell fisheries without special equipment for respiration. Whereas the average human swimmer can submerge for about a minute, practiced sponge divers in the East Indies dive for about two minutes (Hill, 1912). The average dive in the pearl fisheries lasts 50 to 80 seconds (Money, 1929). In Japan a community of divers has long existed in which the women dive while the men operate the boats.

These "ama" make from 60 to 90 dives during a day's work, each dive varying in length up to $2\frac{1}{2}$ minutes as a limit, and they go as deep as 30 meters. It is interesting that the "ama" use inflatable glasses to relieve pressure on the eyes, and nose clips, and that these aids greatly extend their depth range (Teruoka, 1932).

The practice of diving for many generations has not extended the ability of human divers to endure submergence and work for more than the two or three minutes which is possible for any trained athlete, and the extreme capacity for holding the breath after forced breathing and without working is around six minutes. If breathing is stopped without warning, a man can only hold his breath voluntarily for about a minute, and after a few minutes asphyxia is fatal.

Diving animals seem to have much greater ability to hold their breath than man and other land animals, but definite figures are difficult to obtain. A series of definite and apparently reliable observations has been compiled in table 1. These figures are to be compared with the observations upon voluntary apnea of man. The maximal limits of endurance could only be determined by forcible submersion in plain view. Experiments of this sort are reported in table 2 (Bert, 1870). Physiologists hesitate to drown animals for experimentation, although it is common practice to get rid of them in this manner. Domesticated ducks can survive forcible submersion for 10 or 15 minutes (Bert, 1870). A muskrat which had been held under water for nearly 12 minutes, when allowed to emerge, immediately dove again in attempting to escape (Irving, unpublished). A beaver was held under water repeatedly for about six minutes. The beaver's first struggles caused the apprehensive observer to remove his hands, and the duration of submergence was not near the beaver's tolerance (Irving and Orr, 1935).

In substance the observations show that land mammals can without preparation endure voluntary apnea for about a minute, while the divers can submerge for at least ten times as long. The difference apparently places the divers in a different order of capacity for enduring asphyxia. The whales show a different order of endurance from the small divers; for their endurance apparently ranges around an hour. Whether the great endurance of the whales is related to size is not shown. The bottle-nosed whale (Hyperoodon rostratus) is ranked among the ablest at diving; but since it is 20 or 30 feet long, its size is still quite out of the range of common land mammals. For comparison it would be interesting to examine the elephant's tolerance of asphyxia. Unfortunately there are no recorded observations on the diving capacity of

small porpoises, and so it cannot be suggested whether extreme diving capacity is peculiar to whales, or whether it is related to their huge size. Among the diving animals ranging in size between the muskrat (600 grams) and the seal (100 kgm.) there seems to be no relation between

TABLE 1

Duration of dives of diving mammals

ANIMAL	TIME	REFERENCE
Platypus	10 min.	Allport, 1878, p. 30–31 Burrell, 1927, p. 152, 156
Sea elephant (Mirounga angustirostris)	6 min. 48 sec.	Harnisch, 1937, p. 225
Harbor seal (Phoca vitulina)	15 min. (3–6 min. in zoo)	Millais, 1906, vol. 1
Gray seal (Halichoerus grypus)	15 min. in net	Millais, 1906, vol. 1, p. 288
Muskrat (Ondatra zibethica)	12 min.	Irving, unpublished
Beaver (Castor canadensis)	15 min.	Irving and Orr, 1935
Hippopotamus (Hippopotamus amphibius)	50 sec.	Parker, 1932, p. 577
Florida Manatee (Trichechus latirostris)	16 min. 20 sec.	Parker, 1922, p. 127
Sperm whale (Physeter macrocephalus)	1–1½ hours	Millais, 1906, vol. III Scammon, 1874, p. 76 Beddard, 1900, p. 128
Bottled-nosed whale (Hyperoodon rostratus)	2 hours (1 hour wounded)	Gray, 1882, p. 726 Millais, 1906
Bowhead whale (Balaena mysticetus)	1 hour 20 min.	Scammon, 1874, p. 56
Greenland whale	1 hour	Beddard, 1900, p. 128
Common rorqual (Balaena musculus)	49 min.	Millais, 1906, III, p. 268
Blue whale (Balaena sibbaldi)	50 min.	Andrews, 1916
Finback whale (Balaenoptera physalus)	½ hour	Allen, 1916, p. 193
Fin whale (Balaenoptera plupalus)	20 min.	Ommanney, 1932, p. 327
New Zealand humpbacked whale (Megaptera nodosa)	Not over ½ hour	Lillie, 1910, I, Pt. 3

size and endurance. These animals are comparable to man and other laboratory mammals in size, but dissimilar in ability to endure apnea.

The list of diving mammals is not complete and probably should contain representatives from every mammalian order excepting the Eden-

tates, Chiroptera and Primates (Howell, 1930). Although they are phylogenetically diverse, the divers are alike in respiratory ability. This ability is distinct from that of man and land mammals, and it is not acquired by several generations of practice. Nevertheless, the appearance of diving ability in such different orders of mammals suggests that certain fundamental characters of respiration have been highly developed in the divers. On account of the various phylogenies of the divers it is probable that the traces or bases for these developments exist among the respiratory adjustments of all mammals and give to the land mammals the relatively small resistance to asphyxia which they possess. Although small, this endurance nevertheless determines their respiratory habits and is critical for their survival.

We can take man as the type of terrestrial mammal because the human physiological dimensions are well known and we can examine the

TABLE 2*

Endurance of forced submersion

(Average time to last movement)

Dog (3)	4 min. 25 sec.
Cat (3 young)	2 min. 55 sec.
Rabbit (6)	3 min.
White rat	2 min. 6 sec.
Water rat (Arsciola amphibius)	2 min. 17 sec.
Seal	15 min.

* Bert, P. 1870 Physiologie de la Respiration.

details which would count in giving capacity for enduring asphyxia, considering that the store of oxygen is the essential factor. The average man of 70 kgm. would then contain the following quantities of oxygen:

In the lungs—about 6 liters containing about 15 per cent O_2, 900 ml.

In the blood—10 per cent of the body's volume containing not over 18 ml O_2 per 100 ml. blood, 1160 ml.

In the tissue fluids—70 per cent of the body containing 0.005 per cent O_2 if saturated in air, 245 ml. This quantity can be no larger and may actually be very much less. The sum of these stores, which make the greatest possible allowance for oxygen capacity, is about 2300 ml.

The metabolism of a 70 kgm. man would require about 200 ml O_2 per minute under basal conditions, or in 11 minutes of basal metabolism the store of oxygen would be utterly consumed. Under practical con-

ditions, the metabolic use of oxygen during apnea even at rest would be about 50 per cent greater than basal, and a reduction of the reserves of oxygen below 50 per cent cannot be voluntarily tolerated. The limit for voluntary apnea would then be about 4 minutes, which is about the duration possible after forced breathing. The tolerance of apnea conforms with the supply of oxygen, but the influence of other factors is not excluded by the agreement of these figures.

Tolerance of asphyxia by humans is not necessarily typical of mammals, but the dimensions of the various factors involved in metabolism of man are well known, and other less well known mammals can be judged by comparison. It is clear that the diving mammals, which can hold out for about ten times as long as man, are quite distinct from land animals.

This difference might depend upon a smaller requirement of oxygen than that for man. The consumption of oxygen of young resting seals was found to be 96 kgm. cal/sq.m./hr., which was larger than for a child of corresponding age (Irving et al., 1935b). The oxygen requirement of other diving animals has not been determined, but there is no reason to believe that the basal requirement of divers would be smaller than usual.

Since the energy expenditure of mammals varies according to the surface, or the $\frac{2}{3}$ or $\frac{3}{4}$ power of the weight, while the lung volume varies with the weight, large size gives relative to metabolism a greater opportunity for storage of oxygen, which would figure out as about 2800 liters of oxygen in the lungs of a blue whale weighing 122,000 kgm. This store would suffice for 53 minutes at the calculated expenditure of energy necessary to swim at 3 knots (Krogh, 1934). Since experimentation with whales is not practical, these calculations indicate how extrapolation to the size of a whale suggests ability which is quite out of the range of animals of ordinary size. Among the smaller divers the muskrat, weighing 600 grams, endured 12 minutes' forced submersion in the laboratory (p. 114), ducks (2 kgm.) withstand about 15 minutes (Bert, 1870) and the only precise report on a seal (100 kgm.) is about 15 minutes (table 1). The influence of size may be effective in diving ability, but it is not apparent among smaller divers. The whales are credited with the greatest diving power of all mammals, but there are no records of the endurance of small cetaceans which would indicate whether the extreme ability of the whales is due to their large size or to structural or physiological characters which are peculiar to the order.

There is no experimental basis for suggesting that diving animals

diminish their basal metabolic requirements at will or promptly enough in any way to facilitate diving. It was, however, noticed by Paul Bert (1870) that ducks relaxed promptly when submerged and waited without much movement for some minutes. Land animals on the other hand struggle violently as soon as they are forcibly submerged. This muscular relaxation is shown by the muskrat (Irving, unpublished) and beaver (Irving and Orr, 1935) among mammals. Muscular relaxation is a conservational adjustment of these and probably of all divers which avoids wasting the oxygen supply in useless struggles. However, a practiced human diver or athlete by deliberate relaxation can only hold his breath for about five minutes after preparatory re-breathing. The oxygen requirement is then comparable to that of resting metabolism, or is about 30 per cent greater than the basal rate. So it seems that the diving animal could avoid waste by relaxation, but could not suppress its requirement for energy to make the oxygen store pay for fifteen minutes of apnea. The submerged diver is often quite active. Activity probably shortens the duration of a dive, for the degree of activity in long dives is considerably above the resting level.

It has been assumed that the reserves of the diving animal would be comparable to those of man and other land animals, but the dimensions of stored air, blood volume and oxygen capacity might be greater. However, the capacity of the lungs is limited by the common design of mammals, and there is no appearance of an enlargement of thoracic capacity in divers beyond that of active mammals. Examination of lungs of beaver, muskrats, ducks and seals without measurement does not suggest difference in size from the lungs of cats and dogs (Irving, unpublished). Measurement of the air capacity of ducks and chickens gave about the same values (Irving, unpublished). The lungs of blue whales weigh less proportionally than human lungs, but an estimate of capacity corresponds to the proportions of man (Laurie, 1933).

Paul Bert (1870) secured nearly twice as much blood from ducks as from chickens, and the large veins and retia mirabilia of seals, muskrats and beaver suggest the presence of a large volume of blood. Scammon (1874) said that "the quantity of blood (in the sea lion) is supposed to be double that contained in an ox, in proportion to its size." Blood volume determinations on two seals by the dye method showed about 10 per cent blood in the body (Irving, unpublished). It would seem that the blood volume should be carefully determined in diving animals, but it does not seem likely that the blood volume or the volume of air in the lungs could differ radically from the land mammals. Together

the blood and lungs occupy from about 20 per cent of the volume of a mammal. To postulate double their volume would destroy the usual proportions of a mammal, and would appear inconsistent with general structural requirements of the mammalian pattern. The diving animal has from 5 to 10 times the ability of land mammals for enduring asphyxia, so that while the greatest possible enlargement of oxygen capacity could be helpful, it would not be adequate to account for the ability of divers (Irving, 1934).

The blood of diving animals is deeply colored and is suspected of having a large capacity for oxygen. In some cases this is true, as table 3 shows. The greater oxygen capacity is attributable to increased concentration of corpuscles and to a rather large capacity of the corpuscles themselves. The capacity for oxygen of the corpuscles is about a third greater than is usual in land mammals. It would be interesting to extend these observations further in order to determine whether the oxygen capacity of the corpuscles of the various orders of divers is consistently greater. The stress of oxygen want at high altitude or in vascular disease may cause concentration of the corpuscles in human blood, but a prolonged evolutionary influence would be required to modify the concentration of hemoglobin in the corpuscles.

TABLE 3

Oxygen capacity of blood in equilibrium with air

ANIMAL	BLOOD	CELLS	REFERENCE
	ml. O_2/100 ml.	*ml. O_2/100 ml.*	
Seals (11)	29.3	61.3	Irving et al., 1935a
Duck (10)	16.9		Wastl and Leiner, 1931
Man	20.7	45.0	Bock et al., 1924
Dog	21.8		Dill et al., 1932
Sea lion (1)	19.8	68.0	Florkin and Redfield, 1931
Porpoise (1)	20.5	57.7	Green and Redfield, 1933
Porpoise	42.5		Sudzuki, 1924
Beaver (6)	17.7	40.0	Black, unpublished
Muskrat (3)	25.0	50.0	Black, unpublished
Blue and fin whales	14.1		Laurie, 1933

The shape of the oxygen dissociation curves of the divers is characteristic of mammals, and the blood of the seal differs from human blood no more than does the blood of the dog or cat. The same generalization applies to the figures which are given for the transport of carbon dioxide. Carbon dioxide capacity and buffering are less in the blood

of seals than in human blood and more like the blood of the dog (Irving et al., 1935a). In short, if there are peculiarities in respect to transport of oxygen and carbon dioxide by the blood, they are such that any mammal may show and are not attributable to the habit of diving. One might propose that these properties of the blood were established at an early period in the paleontological history of mammals when they were all confirmed land dwellers. When some mammals subsequently returned to an aquatic life they took with them the blood of typical land mammals, and this blood remained unmodified while many solid structures changed radically to conform with the new habitat. A comparison of such stable chemical and physiological properties of animals might serve to date their phylogeny better than the solid forms which are so plastic.

The larger oxygen capacity in the blood of some divers facilitates oxygen transport and increases the store of oxygen. An increase of blood oxygen of 50 per cent with a blood volume of 10 per cent would provide about 500 ml. more of oxygen in a mammal the size of man, a quantity sufficient for two minutes' basal metabolism, or about 15 per cent addition to the reserves. Such a quantity, added to possible enlargement of other stores would be helpful to a diver, but would still not account for a five or tenfold greater endurance of asphyxia.

Suggestions have been made that special stores of oxygen exist in the tissues of diving animals. This view is reminiscent of Pflüger's idea that anaerobic metabolism in tissues used oxygen which was somehow loosely bound, and should be discarded because anaerobic metabolism is now regarded as based primarily upon glycolysis. However, recent studies of tissue respiratory systems propose that muscle hemoglobin has sufficient capacity for oxygen to constitute a significant store of oxygen (Whipple, 1926; Theorell, 1932). Theorell remarked upon the large concentration of the pigment in pressed juice from the muscle of seals to suggest its significance as a store of oxygen for diving. In dog muscle Whipple found about 0.017 gram Fe per kilogram of wet muscle. Since muscle hemoglobin has the same oxygen capacity as hemoglobin, or 400 ml. of oxygen per gram Fe (Barcroft, 1914), the muscles of a dog would contain 6.8 ml. of oxygen per kgm. when saturated with air. Theorell's estimates for the hemoglobin of the dog's heart indicate a similar oxygen capacity. Taking a mammal for reference with dimensions of a 70 kgm. man, the 50 kgm. of soft tissues, excluding blood, would contain with these proportions of muscle hemoglobin capacity for 335 ml. of oxygen when saturated. This is about 34 per cent of the

oxygen store of the blood, or about 10 per cent of the oxygen store of the whole body (p. 116). The affinity of muscle hemoglobin for oxygen is such that 90 per cent saturation would occur at 5 mm. pressure of oxygen at 20°C. (Hill, 1936). Possibly the muscle hemoglobin of a mammal could be nearly saturated at rest, and in that case it would serve an appreciable storage function which would be significant, as Hill (1936) and Millikan (1936) suggest in tiding over the oxygen requirements of the heart from beat to beat. Such a store would, however, be only significant as a store for a fraction of a minute, whereas the endurance of divers requires provision for a number of minutes.

If oxidized cytochrome-c could serve as a store of oxygen, it might (as calculated from the figures of Keilin and Hartree (1937)) have only $\frac{1}{10}$ of the capacity of muscle hemoglobin. One could propose that the concentration of muscle hemoglobin or cytochrome could be great enough in the diving animals to have significance in asphyxia as oxygen stores. But the suggestion is based upon a confusion of the respiratory significance of these pigments, which modify the kinetics of oxygen transport only where small quantities are transported. The respiratory pigments are probably mainly important in divers as in other mammals in the kinetics of respiratory transport and can scarcely be important for storage.

It has frequently been observed that the fat of the diving animals, which is so conspicuous in the blubber of whales and particularly in the spermaceti of the sperm whale is related to diving by dissolving a store of oxygen on account of the large solubility of gases in fats. Although the solubility of oxygen in fat is about five times as great as in water (Laurie, 1933) the fat in a whale does not amount to more than one third of the water, and the storage of oxygen in fat is as small as in the body water. The extra quantity would not be significant for diving.

The estimated oxygen storage of man is given in table 4 along with a second column to indicate the greater capacity which might reasonably be attributed to a diving animal of similar dimensions. The estimates for man (from p. 116) were large allowances for the greatest possible saturation with oxygen. The allowances for a diving animal are about as large as seem to be reasonable without doing violence to its plan of structure and composition as a mammal. While the sum of the additional allowances for divers is significant, it exceeds by only 60 per cent the stores of man, and it indicates that it is unlikely that the factor of enlarged oxygen storage is responsible for the fact that diving animals can submerge for about ten times as long as man.

During brief strenuous activity a man derives energy for muscular activity from anaerobic reactions. The anaerobic provision of energy may be equivalent to that derived from about 20 liters of oxygen (Sargent, 1926), which is three or four times as much as the stored oxygen. The basis for anaerobic muscular metabolism rests in a large measure upon glycolysis and formation of lactic acid. The excised muscles of seals incubated in Na_2HPO_4 solution form about 8 to 10 mgm. of lactic acid per gram (Manery, Welch and Irving, 1935), which is about the same as the glycolytic capacity of rabbits' muscles. The muscles of ducks and hens were also found to have similar glycolytic capacities, and so there is no indication that the divers excel non-divers in respect to maximum possible glycolysis.

TABLE 4

Estimated oxygen storage of man

	MAN	POSSIBLE GREATER CAPACITY OF DIVING ANIMAL OF SAME SIZE
	ml.	*ml.*
Lungs	900	1,500
Blood	1,160	1,600
Fluids	245	245
Tissue respiratory pigments	335	670
Fats		250
Total	2,640	4,265

The amount of lactic acid formed during exercise never reaches the limit of total glycolysis but is apparently checked by acidification of the tissues (Hill and Kupalov, 1929). With this observation in mind it might be proposed that the muscles of divers excel in buffering power and are thus able to endure a greater concentration of acid. No direct determinations of the buffering capacity of muscles of diving animals have been made, but the carbon dioxide content of freshly excised muscles, which represents buffering capacity, is similar to that of land animals in seals and ducks (Manery, Welch and Irving, 1935) and muskrats (Irving, unpublished). These observations are few, but they do not indicate any difference in the glycolytic capacity of the divers which have been examined. It might even be suggested that the anaerobic mechanism in muscles is more primitive or more fundamental in mammals generally than the diving habit which a few members of the class have assumed.

If the anaerobic process in typical mammalian muscle could decrease the utilization of oxygen in the body as a whole it would greatly extend the endurance of asphyxia. But a mammal can apparently only endure apnea as long as some oxygen is available. The anaerobic state in the tissues rapidly absorbs oxygen from the blood as long as any is available, and so would not prevent the depletion of the oxygen of the circulating blood. The characteristic of possessing large reserves for anaerobic metabolism is primarily a property of muscle, and far less of other tissues like brain and heart.

The possibility of increased oxygen storage in the blood has already been examined, and it was remarked that the greatest possible blood volume in divers cannot account for their endurance of asphyxia. A *large volume of blood is not without importance, for it may increase the* rapidity of respiratory transport to a significant degree even though its storage capacity is not significantly large in relation to the requirements of metabolism.

Whalers and anatomists have remarked upon the large size of the veins of diving animals (Hunter, 1787; Houston, 1835; Burow, 1838). In particular the vena cava behind the diaphragm is greatly enlarged in the sea lion (Murie—see Howell, 1930 p. 322), walrus (Burne, 1909), common seal *Phoca vitulina* (Burne, 1910) and hippopotamus (Gratiolet, 1860).

In the diving animals there are frequent ramifications of the arteries and plexuses of veins which greatly complicate the vascular paths. They occur in many animals (Wislocki, 1928, reviews their occurrence and structure), but they are not peculiar to diving animals (Howell, 1930 p. 323). Ommaney (1932) examined these vascular structures in whales. It is not entirely clear whether the anatomical studies of these retia mirabilia have demonstrated what interference with or possible diversion of the circulation they might involve. It is doubtful whether the extra volume of blood which they or the veins contain would contribute significantly to oxygen storage. On the other hand, if they divert or retard the circulation locally, a function which would require some measure of specific local control as well, they could effect great modification in the kinetics of respiratory transport. An examination of the mechanical operation of these "retia mirabilia" is the first prerequisite to indication of their function.

Another interesting vascular structure has been described in the muscular band which surrounds the vena cava at the level of the diaphragm. Gratiolet (1860) observed it in the hippopotamus and proposed that its constriction served as a sphincter to prevent the engorge-

ment of the anterior circulation during asphyxia. Burne has described its occurrence in the seal (1910) and walrus (1909) and Paramore (1910) has discussed its structure and possible function. The structure is reported as a quite distinct muscular band innervated by a separate branch of the phrenic nerve. There is no evidence as to how it operates, but if the sphincter can shut off the venous return through the vena cava, perhaps by damming up the blood in the large abdominal vena cava (p. 123) the resultant alteration of the circulation would be quite profound. When the posterior vena cava is experimentally constricted in ordinary mammals the subsequent vascular adjustments are extensive and varied, but their purposive relation to diving is still too uncertain for speculation until the operation of the sphincter can be actually observed.

These are several peculiarities of the vascular system which suggest that its units have not been well enough defined to permit a description of its integrated action in respiratory transport. It is noticeable, however, that the retia and large veins provide storage capacity which could retain a large amount of blood and which might well keep it temporarily distinct from the blood which is in active circulation.

The cessation of circulation to any part would affect various tissues quite differently. An arm or leg can endure anoxemia for 15 minutes or so without much discomfort when a tourniquet is applied, and no lasting damage is apparent until after an hour. Nerve fibers are likewise quite resistant, but the brain is extremely sensitive. Total ligation of the blood supply to the brain of anesthetized cats caused permanent functional and visible histological damage within a few minutes (Gildea and Cobb, 1930). Occlusion of the common carotids in man promptly causes unconsciousness. Apparently the brain, as a whole will not operate at all during true anoxemia, and the oxygen content of venous blood returning from the brain rarely falls below 10 vols. per cent. The respiratory center, some centers in the cord and some sympathetic ganglia are quite resistant and will continue to discharge rhythmical trains of impulses for some 15 minutes and may be revived after longer asphyxia (Farber, 1936; Heymans et al., 1933; Bronk et al., 1938; Bargeton, 1938). The different duration of survival of the parts of the nervous system indicates various sensitivity to anoxia, but the integrated whole which is necessary to conscious activity is apparently much more delicate than some of the components. Heart muscle may operate anaerobically much as skeletal muscle (Redfield and Medearis, 1926), but the heart as a whole is quickly affected by anoxia. Usually

it is considered that the interconnection between neurones is sensitive to asphyxia, but this is not universally true in view of the survival of the centers and ganglia described above. Nerve fibers are quite resistant to asphyxia, but their peripheral nerve endings are more susceptible and can survive in condition suitable for experimental work during only a few minutes of asphyxia.

Newborn mammals will survive prolonged asphyxia which would be fatal to the adults (Bert, 1870; Avery and Johlin, 1932; Reiss, 1931; Reiss and Haurowitz, 1929). Robert Boyle (1725) remarked that the resistance of kittens to asphyxia was significant in their survival of the difficulty of beginning respiration at birth, a theme which has been subsequently discussed. We do not know what factors in resistance enable the newborn mammal generally to endure asphyxia, but it does not seem likely that divers retain in adult life a hardihood which all other mammals lose. It seems likely furthermore that the sensitivity of the brain to asphyxia is a universal mammalian trait and that any arrangement which preserves the diver must maintain an oxygen supply for the brain and probably for the heart (Irving, 1934). The other tissues are less sensitive to asphyxia and also less indispensable to life.

The addition of carbon dioxide to the inspired air increases blood flow through the brain and reduces the muscular circulation in man (Lennox and Gibbs, 1932), cat (Schmidt, 1928), dog (Irving and Welch, 1935), rabbit, muskrat and beaver (Irving, 1938a). The effect of this vascular adjustment would be to preserve the oxygen in the lungs and blood mainly for the brain at the expense of the muscles, which are, however, quite hardy. For diving animals an adjustment of this sort would appear to be very appropriate but it is a question whether the concentration of carbon dioxide can be raised to the level necessary to effect this vascular reaction before the oxygen supply has been seriously depleted. Furthermore the respiration of seals, muskrats and beaver is relatively insensitive to carbon dioxide and the reduction of muscular blood flow by carbon dioxide does not appear in muskrats and beaver (Irving, 1938b).

It has been reported that when the lungs of beaver are inflated the blood flow through the brain is increased while flow through the muscles diminishes (Irving, 1937). A similar redistribution of the circulation occurs in muskrats, but is not peculiar to divers; for it appears, although less conspicuously, in cats, dogs and rabbits (Irving, 1938a). Arrest of breathing by clamping the trachea or by the cessation of artificial ventilation of a curarized animal is followed by the same vascular

change, and it was suggested that such an adjustment was well arranged to preserve for the brain during apnea the major part of the oxygen store of the body. This adjustment probably occurs less effectively in land animals than in divers, and so it illustrates how a vascular adjustment common to all mammals has perhaps been accentuated in divers to provide for their long endurance of apnea.

The reduction in muscular circulation which follows arrest of breathing is transmitted over the sympathetic nerves (Irving, 1938a). It resembles the effect of carbon dioxide, except that it is more rapid in appearance and it is conspicuous in beaver and muskrat, animals which are not sensitive to carbon dioxide (Irving, 1938b). To allow it definitely to be said that this vascular adjustment is the principal or even an important physiological adjustment of divers requires more specific determination of the method of its control and operation. It remains at least an interesting adjustment to which might reasonably be ascribed critical importance for diving and for the survival of all mammals during apnea.

The heart of divers is retarded during apnea (duck—Bert, 1870; Huxley, 1913; cormorant, swan—Paton, 1927; muskrat—Koppanyi and Dooley, 1929; seal—Irving et al., 1935b; beaver—Irving and Orr, 1935). The retardation may amount to more than 50 per cent of the frequency and diminishes the perceptible strength of the beat. The vagus is apparently necessary for cardiac retardation, and vagotomized or atropinized ducks were no better able than chickens to resist asphyxia (Richet, 1899). The inhibitory influence of the vagus in ducks is very potent, and the heart of an anesthetized duck does not escape from continued electrical vagus stimulation (Battelli and Stern, 1908). In man the slight cardiac retardation often appears during holding the breath (Schneider, 1930). However, one must be cautious in defining the origin of respiratory cardiac arrhythmias. A man holding his lungs inflated is subjected to stimuli from pulmonary stretch receptors; the diver in apnea without inflation still shows a large cardiac retardation. It would seem to be a favorable field in which to investigate among divers the exact control of cardiac arrhythmia, for a retardation of 50 per cent can easily be maintained during apnea for several minutes in a conscious and usually docile animal like the seal or beaver.

Interrupted breathing has been reported to occur in several divers naturally even when out of water; hippopotamus (Swindle, 1926) but not according to Harnisch (1937); seals (Irving et al., 1935b; Harnisch, 1937); sea elephants (Harnisch, 1937). The rhythmically interrupted

breathing of divers has been compared with Cheyne-Stokes breathing. Although the basis of the latter pathological form is not very well defined, it is attributed to a condition developing when sensitivity of the respiration to carbon dioxide is abnormally diminished by pathological depression of the respiratory center. The rhythmical breathing of man at high altitudes is dissimilar at least in that it is not the result of pathological central depression, and the rhythmical breathing of seals is certainly not pathological. It is pertinent that the breathing of the divers, as far as they have been examined, is not easily affected by carbon dioxide. Orr and Watson (1913) remarked upon the insensitivity of the duck, and the breathing of seals (Irving et al., 1935b), beaver and muskrats (Irving, 1938b) is not much increased by the inhalation of 10 per cent carbon dioxide. In general it is considered that the respiratory center of mammals is stimulated by carbon dioxide, while the chemoreceptors in the aorta and carotid body are more sensitive to lack of oxygen (Comroe and Schmidt, 1938). According to this picture, which was suggested to me by Doctor Schmidt, it wouldbe expected that the diving animals, which are insensitive to carbon dioxide, regulate their respiration mainly through the peripheral chemoreceptors rather than through the activity of the center, which is the predominant control in non divers.

It should be remarked upon that the significant factor which is involved in stimulation by carbon dioxide or lack of oxygen is not immediately obvious. As an example, we may consider that mammalian breathing is not appreciably affected until the oxygen content of the alveolar air is reduced to about 12 vol. per cent. Further reduction soon leads to asphyxia. Raising alveolar carbon dioxide by only 1 mm. greatly increases breathing, and rising carbon dioxide tension steadily increases ventilation. These effects are illustrated in figure 9 of Comroe and Schmidt's (1938) paper, in which it is seen that the effectiveness of carbon dioxide in the carotid body upon breathing duplicates the gradual rise with pressure of a carbon dioxide dissociation curve of blood; the sudden effect of oxygen lack resembles the sudden decline of an oxygen dissociation curve. The capacity of the blood for oxygen and carbon dioxide is interposed between the alveoli and the chemoreceptors, and the eventual pressure of gas in the sensory region is modified by the buffering or capacity of the blood. For these reasons, at least, it is not possible to say that the insensitivity of the divers to carbon dioxide represents immediately a peculiarity of the chemoreceptors, for the pressures which are measured during inhalation are still

quite remote from the sensory regions which are stimulated. Capacity factors in the system for respiratory transport determine what final pressure will exist in the tissues, and these capacity factors are not yet known in either the diving or non diving mammals.

The periodically interrupted breathing of the seal presents a striking natural analysis of the physiological components of respiration (Irving et al., 1935b; Irving, 1936). Breathing occurs in intermittent groups of from 4 to 6 breaths separated by intervals lasting from 20 to 40 seconds. As soon as breathing ceases, the heart begins to slow, decreasing in the period of three or four beats from a frequency of 70 to 90 to a frequency of 35 to 50. Under anesthesia it may also be observed that the mean arterial blood pressure regularly rises during apnea at the time when the heart slows. The pressure rise is, then, the result of peripheral vasoconstriction which must be quite intense in view of the decreased frequency and amplitude of the heart beat. The peripheral site of the vasoconstriction has not yet been determined in the seal, but in the beaver and muskrat, whenever breathing ceases, the blood flow through the muscles decreases greatly, while flow through the brain increases (Irving, 1937, 1938a). This picture of the coördination of the factors in respiration probably indicates that the principal protection of the animal as a whole against asphyxia is accomplished by the appropriate differentiation of the system of respiratory transport.

This differentiation is delicately coördinated in timing and in the peripheral field which is covered. It certainly involves changes in blood flow through brain and muscles, and probably in the liver (Irving, unpublished). Periodic breathing provides ideal conditions for the demonstration of the correlation of breathing, cardiac and peripheral vascular condition. The opportunity is also presented for estimating the relative independence of the central mechanisms which control breathing, the heart, and blood flow. At first sight breathing seems to set the pace, but there is evidently some physiological distinction between the three centers. Administration of carbon dioxide to a seal increased the amplitude of breathing tremendously and frequency slightly. The heart was generally retarded, and blood pressure diminished only slightly during the pauses in breathing. Breathing stopped spontaneously, and changes in the heart and blood flow continued to occur and were not entirely suppressed by artificial ventilation. Purposive significance is not so apparent nor interesting in these observations as is the fact that the periodic breathing of the seal and its range of cardiac and peripheral

vascular changes afford a fine opportunity for analyzing the system of respiratory control and integration.

It was noticed quite early that the submerged diving animal (duck—Bert, 1870) relaxes muscular activity. In contrast to the violent struggling which a land animal makes when suddenly and forcibly immersed in water or when prevented in any way from breathing, the diving animal accepts the situation with equanimity and waits with muscles relaxed for several minutes. It then usually deliberately explores the means for escape, and often does not begin violent struggling until five or ten minutes have elapsed. This behavior is true for the muskrat (Irving, unpublished), beaver (Irving and Orr, 1935) and seal (Irving et al., 1935b). It may require conditioning, for Richet (1898) reported that ducks raised away from the water would survive their first immersion for from 5 to 15 minutes, while after training the time was extended to from 12 to 17 minutes.

The decerebrate duck will still show apnea when the head and neck are straightened with beak slightly elevated, and retardation of the heart accompanies the apnea (Huxley, 1913). Extirpation of the labyrinths decreases the certainty and duration of the apnea, but the proprioreceptors of the cervical muscles are evidently essential for apnea (Huxley, 1913; Paton, 1913). It seems rather strange that respiratory reflexes should be elicited by the stimulation of typical postural proprioceptors. The association is probably not peculiar to the duck among diving animals. Most of the other respiratory adjustments of divers are only quantitatively but not qualitatively different from those of land animals, and it may be that the influence of proprioceptors upon the respiratory center (including cardiac and vasomotor control) is based upon a fundamental nervous connection between the two systems. There is in fact some experimental evidence for a specific influence of the VIIIth nerve upon the circulation of dogs and rabbits (Camis, 1912; Spiegel and Démétriades, 1924; Mies, 1936).

There has been much speculation as to how whales in their deep dives escape the danger of caisson disease. One of the most interesting suggestions was that nitrogen fixing bacteria in the blood of whales could remove nitrogen fast enough to assist the whale against danger of caisson disease. The presence of nitrogen fixing bacteria in the blood of whales was reported (Laurie, 1933), but such a surprising symbiotic relationship can only be considered when it is more clearly demonstrated. There is, of course, no experimental data on the subject of caisson dis-

ease in whales but whales are not known to suffer in this way, and in fact, it seems unlikely that they would, because they do not rebreathe air in a caisson (Howell, 1930; Irving, 1935). Four atmospheres' pressure would force all of the nitrogen in whales' lungs into solution in tissues. The quantity dissolved would be about three times greater than a human diver is supposed to tolerate for immediate decompression. The whale, however, swims gradually and not instantly down and up, and its activity favors the elimination at the same rate as the solution occurred during the dive. Any considerable excess of the rate of solution of nitrogen over the rate of its escape during repeated diving would gradually cause the whale's tissues to become charged with nitrogen; but there is no evidence that the disparity occurs.

There are doubtless other factors than those mentioned which are concerned with the endurance of asphyxia, but the essential physiology seems clear. The storage of oxygen is inadequate to provide for its indiscriminate use by all of the tissues. No shift of metabolism can compensate for the lack of oxygen, but differential control of the distribution of oxygen might reasonably serve to maintain the brain, allowing the less sensitive tissues or those with fair capacity for anaerobic metabolism to do without oxygen. Effective control of the circulation does reduce blood flow through the muscles and increase the flow through the brain. Incidental control of the heart and the suppression of superfluous muscular activity assists the effectiveness of the adjustments.

Taken together, these physiological functions could reasonably provide the resistance to asphyxia which divers show. In the smaller degree of their appearance in all mammals, the adjustment of respiration determines their lesser endurance of asphyxia, and although the time is shorter, it is nevertheless of critical importance.

It is interesting that the principal physiological adjustments of divers differ only quantitatively from those of land mammals. Physiological characters are often considered to be quite plastic toward environmental influences, while anatomical structures are said to be fixed. But the principal respiratory functions of the mammals persist without essential alteration after the long stress of aquatic life. It is only in a quantitative way that conservational adjustments have become accentuated.

The accentuation of certain fundamental physiological traits in the divers provides a valuable set of natural experiments in the physiology of respiration. Particularly in the control of respiration, the diving animals demonstrate clear signs of central activity which are indistinct in other animals, but which in the divers should provide the opportunity

for finer analysis than could possibly result from the consideration of land animals alone.

REFERENCES

*ALLEN, G. M. The whalebone whales of New England. Mem. Boston Soc. Nat. Hist. 8: 193, 1916.

*ALLPORT, M. Notes on the Platypus. Report of the Roy. Soc. Tasmania, p. 30, 1878.

*ANDREWS, R. C. Whale hunting with gun and camera. New York, 1916.

AVERY, R. C. AND J. M. JOHLIN. Relative susceptibility of adult and young mice to asphyxiation. Proc. Soc. Exper. Biol. Med. 29: 1184, 1932.

BARCROFT, J. The respiratory function of the blood. Cambridge, 1914.

BARGETON, D. Acute anemia and transmission of impulses through sympathetic ganglion. Am. J. Physiol. 121: 261, 1938.

BATTELLI, E. AND L. STERN. Excitabilité du nerf vague chez le canard. C. R. Soc. de Biol. 65: 505, 1908.

*BEDDARD, F. E. Book of whales. New York, 1900.

BERT, P. Physiologie de la Respiration. Paris, 1870.

BLACK, E. C. *Unpublished.*

BOCK, A. V., H. FIELD, JR. AND G. S. ADAIR. The oxygen and carbon dioxide dissociation curves of human blood. J. Biol. Chem. 59: 353, 1924.

BOYLE, R. The philosophical works of Boyle. London, 1725.

BRONK, D. W., M. G. LARRABEE, J. B. GAYLOR AND F. BRINK, JR. The influence of altered chemical environment on the activity of ganglion cells. Am. J. Physiol. 123: 24, 1938.

BURNE, R. H. Notes on the viscera of the walrus, Octobaenus rosmarus. Proc. Zool. Soc. London, p. 732, 1909.

BURNE, R. H. Note on the veins of a seal. Proc. Zool. Soc. London, p. 385, 1910.

BUROW, C. A. Ueber das Gefässsystem der Robben. Arch. f. Anat. Physiol. (Müller), 230, 1938.

*BURRELL, H. The Platypus. Sydney, 1927.

CAMIS, M. Contributo alla fisiologia del labirintectomia nel cane particolarmente sulla innervazione vasomotoria. Folia Neurobiologica 6: 138, 1912.

COMROE, J. H. AND C. F. SCHMIDT. The part played by reflexes from the carotid body in the chemical regulation of respiration in the dog. Am. J. Physiol. 121: 75, 1938.

DILL, D. B., H. T. EDWARDS, M. FLORKIN AND R. W. CAMPBELL. Properties of dog blood. J. Biol. Chem. 95: 143, 1932.

FARBER, S. Resistance of spinal vasomotor centres to anaemia or asphyxia. J. Physiol. 87: 31P, 1936.

FLORKIN, M. AND A. C. REDFIELD. On the respiratory function of the blood of the sea lion. Biol. Bull. 61: 422, 1931.

GILDEA, E. F. AND S. COBB. The effects of anemia on the cerebral cortex of the cat. Arch. Neurol. Psychiat. Chicago 23: 876, 1930.

* Includes references given in table 1.

GRATIOLET, M. P. Recherches sur le système vasculaire sanguin de l'Hippopotame. C. R. Acad. Sci. Paris 51: 524, 1860.

*GRAY, D. Notes on characteristics and habits of the bottle-nosed whale. Proc. Zool. Soc. London, p. 726, 1882.

GREEN, A. A. AND A. C. REDFIELD. On the respiratory function of the blood of the porpoise. Biol. Bull. 64: 44, 1933.

*HARNISCH, O. Beobachtungen und Gedanken über den Atemrhythmus der Pinnepedier. Zool. Anz. 117: 225, 1937.

HEYMANS, C., J. J. BOUCKAERT AND P. REGNIER. Le sinus carotidien. Paris, 1933.

HILL, A. V. AND P. S. KUPALOV. Anaerobic and aerobic activity in isolated muscle. Proc. Roy. Soc. London, 105: 313, 1929.

HILL, L. Caisson sickness and the physiology of work in compressed air. London, 1912.

HILL, R. Oxygen dissociation curves of muscle haemoglobin. Proc. Roy. Soc. London, B 120: 472, 1936.

HOUSTON, J. On the peculiarities of the circulating organs in diving animals. Brit. Assoc. Adv. Sci. Reports, part 2, p. 81, 1835.

HOWELL, A. B. Aquatic mammals. Springfield, 1930.

HUNTER, J. Observations on the structure and economy of whales. Phil. Trans. Roy. Soc. London 77: 371, 1787.

HUXLEY, F. M. On the reflex nature of apnoea in the duck in diving. Quart. J. Physiol. 6: 147, 1913.

IRVING, L. On the ability of warmblooded animals to survive without breathing. Sci. Mon. 38: 422, 1934.

The protection of whales from the danger of caisson disease. Science 81: 560, 1935.

A demonstration of the respiratory control of seals. The Collecting Net 11: 204, 1936.

The respiration of beaver. J. Cell. and Comp. Physiol, 9: 437, 1937.

Changes in the blood flow through the brain and muscles during the arrest of breathing. Am. J. Physiol. 122: 207, 1938a.

Control of respiration in diving animals. Am. J. Physiol. 123: 107, 1938b.

Unpublished.

*IRVING, L. AND M. D. ORR. The diving habits of the beaver. Science 82: 569, 1935.

IRVING, L., O. M. SOLANDT, D. Y. SOLANDT AND K. C. FISHER. Respiratory characteristics of the blood of the seal. J. Cell. and Comp. Physiol. 6: 393, 1935a.

The respiratory metabolism of the seal and its adjustment to diving. J. Cell. and Comp. Physiol. 7: 137, 1935b.

IRVING, L. AND M. S. WELCH. The effect of the composition of the inspired air on the circulation through the brain. Quart. J. Physiol. 25: 121, 1935.

KEILIN, D. AND E. F. HARTREE. Preparation of pure cytochrome c from the heart muscle and some of its properties. Proc. Roy. Soc. London, B 122: 298, 1937.

KOPPANYI, T. AND M. S. DOOLEY. Submergence and postural apnea in the muskrat. Am. J. Physiol. 88: 592, 1929

KROGH, A. Physiology of the blue whale. Nature 133: 635, 1934.

LAURIE, A. H. Some aspects of respiration in blue and fin whales. Discovery Reports 7: 363, 1933.

LENNOX, W. G. AND E. L. GIBBS. The blood flow in the brain and the leg of man and changes induced by alteration of blood gases. J. Clin. Invest. 11: 1155, 1932.

*LILLIE, D. G. British Antarctic Expedition (Terra Nova). Brit. Mus. Nat. Hist. Reports 1: part 3, 1910.

MANERY, J. F., M. S. WELCH AND L. IRVING. The postmortal formation of lactic acid in the muscles of seals, ducks and hens. J. Cell. and Comp. Physiol. 7: 131, 1935.

MIES, H. Labyrinth und Blutdruckzügler. Ztschr. f. Biol. 97: 219, 1936.

*MILLAIS, J. G. The mammals of Great Britain and Ireland. 1906.

MILLIKAN, G. A. The kinetics of muscle haemoglobin. Proc. Roy. Soc. London, B 120: 366, 1936.

MONEY, SIR L. C. Pearl fisheries. Encycl. Brit. 17: 421, 1929.

*OMMANEY, F. D. The vascular networks (Retia mirabilia) of the fin whale. Discovery Reports 5: 327, 1932.

ORR, J. B. AND A. WATSON. Study of the respiratory mechanism in the duck. J. Physiol. 46: 337, 1913.

PARAMORE, R. H. The evolution of the pelvic floor in the non-mammalian vertebrates and pronograde mammals. Lancet 1: 1459, 1910.

*PARKER, G. H. The breathing of the Florida Manatee (Trichechus latirostris). J. Mammalogy 3: 127, 1922.

The normal period of submergence for the hippopotamus. Am. J. Physiol. 99: 577, 1932.

PATON, D. N. The relative influence of the labyrinthine and cervical elements in the production of postural apnoea in the duck. Quart. J. Exper. Physiol. 6: 197, 1913.

Submergence and postural apnoea in the swan. Proc. Roy. Soc. Edinburgh 47: 283, 1927.

REDFIELD, A. C. AND D. N. MEDEARIS. The content of lactic acid and the development of tension in cardiac muscle. Am. J. Physiol. 77: 662, 1926.

REISS, M. Das Verhalten das Stoffwechsels bei der Erstickung neugeborener Ratten und Mäuse. Ztschr. f. d. ges. exper. Med. 79: 345, 1931.

REISS, M. AND F. HAUROWITZ. Über das Verhalten junger und alter Tiere bei Erstickung. Klin. Wchnschr. 8: 743, 1929.

RICHET, C. De l'influence de l'éducation sur la résistance du canard à l'asphyxie. C. R. Soc. Biol. 50: 481, 1898.

De la résistance des canards à l'asphyxie. J. Physiol. et Path. gen. 1: 641, 1899.

ROBERTSON, L. Saturday Evening Post, p. 10 (June 22), 1935.

SARGENT, R. M. The relation between oxygen requirement and speed in running. Proc. Roy. Soc. London, B 100: 10, 1926.

*SCAMMON, C. M. Marine mammals of the northwest coast of North America. San Francisco, 1874.

SCHMIDT, C. F. The influence of cerebral blood flow on respiration. Am. J. Physiol. 84: 202, 1928.

SCHNEIDER, E. C. Observations on holding the breath. Am. J. Physiol. **94**: 464, 1930.

SHILLING, C. W., R. A. HANSEN AND J. A. HAWKINS. The effect of increased air pressure on vital capacity, expiratory force and breath-holding ability. Am. J. Physiol. **110**: 616, 1934.

*SCORESBY, W., JR. An account of the arctic regions with a history and description of the northern Whale-fishery. Edinburgh. 2 vol., 1820.

SPIEGEL, E. A. AND TH. D. DÉMÉTRIADES. Beiträge zum Studium des vegetativen Nervensystems. VII. Pflüger's Arch. **205**: 328, 1924.

SUDZUKI, M. Untersuchungen über Cetacea. VIII. Uber das Blut. Tohoku J. Exper. Med. **5**: 419, 1924.

SWINDLE, P. F. Superimposed respirations on Cheyne-Stokes breathing of amphibious and non-amphibious mammals. Am. J. Physiol. **79**: 188, 1926.

TERUOKA, G. Die Ama und ihre Arbeit. Arbeitsphysiol. **5**: 239, 1932.

THEORELL, A. H. T. Kristallinsches Myoglobin. I. Biochem. Ztschr. **252**: 1, 1932.

WASTL, H. AND G. LEINER. Beobachtung über die Blutgase bei Vögeln. Pflüger's Arch. **227**: 367, 1931.

WISLOCKI, G. B. Observations on the gross and microscopic anatomy of the sloths (Bradypus griseus griseus Gray and Choleopus hoffmanni Peters). J. Morph. **46**: 317, 1928.

WHIPPLE, G. H. The hemoglobin of striated muscle. Am. J. Physiol. **76**: 693, 1926.

PHYSIOLOGICAL REVIEWS

VOL. 19 APRIL, 1939 No. 2

RESPIRATION IN THE CENTRAL NERVOUS SYSTEM

J. H. QUASTEL

Biochemical Laboratory, Cardiff City Mental Hospital, Cardiff, Wales

Effects of oxygen lack on the central nervous system. The study of respiratory processes in the central nervous system is of more than ordinary interest. This is not only because metabolism in the brain presents a number of unique features, but because there appears to be a close association between oxidation processes in the brain and cerebral activity.

It is known that the gray matter of brain has a high rate of oxygen consumption and that a continuous supply of blood to the brain is essential for the normal functioning of this organ. An interruption of cerebral circulation for six to eight seconds will, for example, produce loss of consciousness. The usual action potentials in the cat's cortex are obliterated by a cerebral anemia of twenty seconds (Simpson and Derbyshire, 1934) and an anemia varying from two to fifteen minutes frequently causes irreversible changes in the cerebral cortex and medulla. The effects of cerebral anemia have been frequently described (e.g., Gildea and Cobb, 1930) and the valuable review of Wolff (1936) on the cerebral circulation gives much useful information on the consequences of decreased oxygen supply to the brain.

Lennox, Gibbs and Gibbs (1935) studied the cerebral blood flow and oxygen saturation of the blood returning from the brain in twenty-two unanesthetized human subjects with reference to loss of consciousness due to 1, syncope; 2, breathing of nitrogen; 3, pressure on the carotid sinus. Oxygen saturation of the blood leaving the brain through the internal jugular veins was measured. Their results make it clear that unconsciousness supervenes if the oxygen supply to the brain is suddenly reduced to such an extent that oxygen saturation of the blood in the internal jugular vein falls to 24 per cent or less. With a saturation value of 30 per cent or more the person remains conscious. It was also found that pressure on the carotid sinus brings about unconsciousness

without a preliminary cerebral anemia or fall in cerebral blood flow. There is no decrease in total cerebral blood flow during sleep (Gibbs, Gibbs and Lennox, 1935) or in epileptic seizures (Lennox and Gibbs, 1936; Gibbs, Lennox and Gibbs, 1934). It is evident, therefore, that although the unconscious state may take place as a consequence of lowering the oxygen supply to the brain it may also occur when oxygen is as freely available to the cerebral tissues as in the conscious condition.

Lennox (1936) impressed with the importance of an abundant oxygen supply to the brain thought it probable that the brain would be amply supplied with capillaries, since "the number of capillaries per unit of tissue is usually a gauge of the metabolic activity of that tissue" (see also Wolff, 1936), and that blood leaving the brain would remain rich in oxygen. It had been shown, however, by Cobb and Talbot (1927) that the number of capillaries of gray matter is small compared with that of muscle, and Lennox found that the residual oxygen saturation of the blood in the internal jugular vein on leaving the brain in conscious humans was approximately 60 per cent. This value was definitely lower than the oxygen content of venous blood from the arm (72 per cent). This points either to a relatively high consumption of oxygen by the tissues of brain, or to a smaller rate of flow of blood through the brain. Wolff (1936), however, considers that the brain circulation time does not differ essentially from that of other organs. Lennox further showed from a study of the distribution of the values of the oxygen saturation of samples of blood from the internal jugular vein, from a vein in the arm and from the femoral vein, that the speed of blood flow in the brain is more constant than in the extremities and suggests that a constant supply of oxygen to the brain is of greater importance than a rich supply (see also Cobb and Forbes, 1937). Acute anoxemia, it is pointed out (Lennox, 1936), may produce nervous symptoms not observed in states of chronic oxygen lack. While it is probably quite true that a constant, rather than a rich, supply of oxygen to the brain is essential to the normal functioning of this organ, it may be suggested that this is related to the fact that the brain is very restricted in its choice of metabolites for respiratory purposes. As will be shown later the brain makes its chief demand for respiratory activity on glucose. Hence a constant respiratory activity of the brain will depend on the availability of at least a minimum concentration of glucose and on a constant supply of oxygen. It is evident that the respirations of organs which have a "mixed" metabolism will fluctuate very considerably with changes in the concentrations of the various metabolites

attacked by these organs and it seems unlikely that variations of oxygen tension will produce such serious consequences in the functioning of these organs as in the case of brain which is so much more restricted in its choice of metabolites.

Experiments in which the circulation of dogs is completely stopped and, after varying intervals, re-initiated have been carried out by Heymans and Bouckaert (1935). After 30 minutes of anoxemia neurones in the brain can still regain their function (respiration, vasomotor and pupillary reflexes). Oxygen lack longer than five minutes results in the cessation of cerebral function but within this interval full recovery (except for blindness) can take place. Various parts of the nervous system differ obviously in their sensitivities to oxygen lack and abilities to recover.

It has been shown by Lennox that the seizures of epileptic patients can be regularly induced by administration of air deficient in oxygen. An attack of petit mal would occur when the oxygen tension of the respired air had fallen to 8–12 per cent whereas unconsciousness does not supervene in normal persons until the oxygen content is reduced to 7 per cent. Conversely an increased oxygen pressure will diminish the number of seizures in an epileptic patient (Lennox and Behnke, 1936), but it is to be noted that high oxygen pressures will themselves induce convulsions. This had already been noted by Bert in 1878, and Shilling and Adams (1933) found that rats confined to oxygen filled chambers would all convulse if the oxygen pressure was raised to $4\frac{1}{2}$ atmospheres. No convulsions took place at a pressure of 3 atmospheres.

It is known that under certain conditions of respiratory depression the administration of oxygen may further depress respiration, probably by removal of an anoxemic stimulus (e.g., CO_2) to respiration. Information concerning these phenomena, a description of which is not strictly relevant to the subject of this review, may be found in the papers of Loevenhart and his colleagues (1918, 1922), d'Elseaux and Solomon (1933) and Marshall and Rosenfeld (1936).

Psychological consequences of oxygen lack. Many of the consequences—both physiological and psychological—of oxygen want have been described by Barcroft (1934, 1936), and by McFarland(1932, 1937) and will not be considered here in any detail. Reference may be made, however, to the recent work of McFarland, Knehr and Berens (1937) who have shown that under anoxemia there is a decrease in efficiency of ocular movements. This is attributed to a diminished amount of oxygen being delivered to the nervous tissue, subcortical as well as

cortical. They consider that the changes in eye movements can be used to detect early effects of oxygen deprivation. Gellhorn (1936) has studied the influence of administration of 3 per cent CO_2 on the effects of oxygen deficiency on visual intensity discrimination. Oxygen deficiency (breathing 8 per cent–9 per cent oxygen) produces a marked decrease in visual discrimination and this is completely offset by 3 per cent CO_2, which itself has no effect on this sensory function. Gellhorn considers that the alleviating effect of the CO_2 is due to the circulatory improvement induced by CO_2 under oxygen deficiency. He points out (Gellhorn, 1937) that 3 per cent CO_2 improves oxygenation of the tissues in general and of the brain in particular by

1. An increase in the respiratory volume.
2. The right shift in the oxygen dissociation curve (Barcroft, 1928).
3. The improved venous return due to the increased muscular tonus (Henderson *et al.*, 1936).
4. The specific dilatory action of CO_2 on the blood vessels of the brain (Cobb and Fremont-Smith, 1931; Gibbs *et al.*, 1935b).
5. The augmentation of rise in blood pressure which occurs under conditions of oxygen want (Lambert and Gellhorn, 1937).

It is interesting to note the fact (Gellhorn and Joslyn, 1936) that oxygen deficiency (breathing 7 per cent–9 per cent O_2), hyperpnea and carbon dioxide excess (6 per cent–7 per cent CO_2) have marked prolonging effects on the time required to accomplish simple mental tests such as number cancellation and the Kraepelin additions test. The results are explained on the basis of insufficient oxygen supply to the cortex. Kraines (1937), too, has shown that oxygen deficiency (breathing 10 per cent O_2) leads to a diminution of intellectual function.

Oxygen consumption by brain in vivo. The data for determinations of the oxygen uptake of brain *in vivo* are not very accurate owing to the obvious experimental difficulties of ascertaining the amount of blood going to the brain, etc. Some approximate estimations may be given.

Himwich and Nahum (1932), recalculating the results of Alexander and Cserna, find that 13 ml. oxygen are consumed per 100 grams dog brain per minute. This gives a Q_{O_2}[1] (μl. O_2 consumed per milligrams dry weight of tissue per hour) of 39 $\left[\text{i.e., } \frac{13000 \times 60}{20000}\right]$ taking the dry weight of brain to be 20 per cent of the wet weight. Hou (1926) calculated that per 100 grams dog brain 5.6 to 7.8 μl O_2 were utilized per minute.

[1] This value, following Warburg, is usually negative since it indicates an absorption of O_2. The negative sign will, however, be omitted in this article.

This gives a Q_{O_2} of 16.8 to 23.4. From values quoted in Page's *Chemistry of the Brain*, p. 326, the Q_{O_2} of rabbit brain *in vivo* is 28.2 (Yamakita) and of dog brain 29.7 (Gayda).

According to Lennox (1936) the oxygen content of blood leaving the human brain is about 60 per cent saturation and is less than that of blood leaving the face and the extremities. The following table 1, taken from his paper, shows his measurements.

There would appear to be a loss of approximately 8 ml. O_2 per 100 ml. of blood in its passage through the human brain. The estimated flow

TABLE 1

Average gaseous composition of blood leaving the face, brain, arm and leg of man

VEIN	O_2 CONTENT PER CENT BY VOLUME	O_2 CAPACITY PER CENT BY VOLUME	O_2 PER CENT SATURATION	CO_2 CONTENT PER CENT VOLUME
Internal jugular	12.0	19.4	61.5	54.7
Antecubital	13.0	20.3	64.2	52.8
Femoral	13.3	19.2	69.2	52.6
External jugular	17.8	20.5	87.0	51.2

of blood through a human brain is about one liter per minute (Schneider and Schneider, 1934)[2]. Taking the weight of the brain as 1400 grams the Q_{O_2} is

$$\frac{80{,}000 \times 60}{280 \times 1000} = 17.1$$

These figures are given to show the order of magnitude of Q_{O_2} of brain *in vivo;* they cannot be regarded as accurate.

Respiratory quotient of brain in vivo. The respiratory quotient of brain *in vivo* is approximately unity. Lennox (1931) compared the O_2 and CO_2 contents of arterial blood with those of the blood in the internal jugular vein, and obtained an R. Q. of 0.95. This quotient is definitely higher than that of the arm (0.86) and of the leg (0.72). Lennox pointed out the limitations of determinations of the respiratory quotient by the arterio-venous difference. The CO_2 content of the blood depends not only on changes in tissue metabolism but on variations in respiratory rate, on the rate of diffusion of CO_2 through the skin and on the acid base composition of the blood. Induced hyperpnea

[2] The flow through rabbit brain is 60 ml. per 100 grams per minute (Winterstein, 1935).

and breathing CO_2 bring about apparent changes in the R. Q. of brain and Lennox arrived at an average R. Q. of 0.95 for human brain on the basis of a large number of experiments. Himwich and Nahum (1932) examined a series of dogs, some of which were anesthetized with amytal, others phlorizinized and some depancreatized and found the R. Q. of the brain *in situ* to be unity.

Such a respiratory quotient points to the probability that carbohydrate is the main fuel of the brain *in vivo*. Myerson and Halloran (1930, 1931) and Lennox (1931) have shown that blood on passing through the brain loses carbohydrate. The difference between the glucose contents of arterial and cerebral venous blood (9 mgm. per cent) is greater than that between the glucose contents of arterial and muscle venous blood (arm 5 mgm. per cent, leg 4 mgm. per cent). Himwich and Fazekas (1937) have examined cerebral blood samples from the superior longitudinal sinus and arterial samples from the femoral arterial. They find the average difference between the glucose contents of these arterial and venous bloods to be 13 mgm. per cent, the animals used being dogs anesthetized with amytal.

If we take the value 10 mgm. per 100 ml. blood to be a roughly accurate measure of the glucose consumption of human brain *in vivo*, then the amount of oxygen required to burn this amount of glucose completely to CO_2 and H_2O would be 7.5 ml. (at N.T.P.). This value corresponds fairly closely to the fall in oxygen content per 100 ml. blood on passing through the brain (see table 1). Again in the experiments of Himwich and Fazekas (1937) the glucose consumption by the brain of amytalized dogs is 13 mgm. per 100 ml. blood. This corresponds to an oxygen consumption of 9.7 ml. per 100 ml. blood—assuming complete oxidation of glucose. They found that the average difference between the arterial and cerebral venous blood was 9.3 ml. per cent. Gerard and Schachter (1932) have made similar experiments and calculations.

These observations make it highly probable that glucose oxidation is at any rate quantitatively the most important respiratory process in the brain *in vivo*.

Respiration of brain in vitro. Much information on this topic[3] is now available and reference may be made to existing reviews of the subject (Page, 1937; Winterstein, 1932; Gerard, 1937; Holmes, 1935).

Manometric methods of Barcroft and of Warburg have been usually

[3] Gerard (1932) has reviewed the subject of nerve metabolism so far as 1932. Further information, after that time, may be found in the Annual Reviews of Biochemistry.

adopted for the estimation of the amounts of oxygen consumed by brain tissue *in vitro*. Details of these methods will not be described here; they may be found in the publications of the workers in this field. In particular the reader may be referred to the papers of Warburg (1930) and his colleagues in which the history of manometric methods, the technical details and the conditions under which such methods should be adopted are given in detail. In addition the reader may be referred to a publication of M. Dixon (1934).

Brain tissue is usually examined in a minced or chopped condition or a tissue brei, and in the form of thin slices usually of cerebral cortex. The tissue is suspended in a suitable medium and measurements of oxygen uptake are made. Oxygen instead of air is present in the manometric vessels when slices are used and the slices must be of suitable dimensions (0.2–0.4 mm. thick) to ensure adequate diffusion of oxygen and not so fragile that they are distintegrated at the termination of the experiment.

A number of workers have considered that so far as the oxidative mechanisms of brain are concerned the process of chopping has little deleterious action. Thus Ashford and Holmes (1931) compared the oxygen uptake of intact freshly dissected frog's brain with that of chopped frog's brain and found that the total oxygen uptake of the chopped brain in four hours at 19° was 16 per cent less than that of the intact tissue. This suggests that chopping brain brings about little effect on the respiration. According to M. Dixon and Elliott (1929) the rates of respiration of sliced and minced tissues are the same and it is assumed that the process of slicing according to Warburg's technique (1923)—with a razor moistened with Ringer solution—does the least possible damage to the tissue. Holmes (1930) thinks that so far as the oxygen uptake of the brain cortex is concerned "it matters little whether it is chopped or sliced." This view is supported to some extent by the evidence of Quastel and Wheatley (1932) who find that when chopped guinea-pig brain is allowed to take up oxygen for two or three hours and the rate of oxidation has fallen considerably owing to depletion of metabolites, the addition of glucose restores the rate of oxidation to a value not remotely removed from the initial level. Had the processes of chopping and shaking the tissue brei in a glucose free phosphate saline medium for 2 or 3 hours at 37° brought about a serious disturbance in the oxidative mechanism, the observed restoration of oxygen uptake on addition of glucose would not have been expected. It is abundantly clear in the case of another organ, liver, that the process of chopping

very greatly affects oxidative systems—for in this case the oxidation of fatty acids, which takes place at a rapid rate with liver slices, is almost completely absent in a liver *brei* (Quastel and Wheatley, 1933).

Thus the evidence points to a comparative stability of oxidative mechanisms in brain or to a comparatively high resistance of brain cells to mechanical injury.[4] It is clear, however, that the process of mincing does involve changes which should be considered in any quantitative work with brain. For example, when minced brain, which has respired in a glucose medium, is centrifuged and the centrifuged deposit placed in a fresh glucose medium, there is a drop of 40 per cent in the rate of respiration. When brain slices, on the other hand, which have also respired in a glucose medium, are removed from this medium, washed in a Ringer medium and placed in a fresh glucose medium, the subsequent rate of respiration does not show a fall in respiration greater than 8 per cent (Quastel and Wheatley, 1934). It would appear that a factor, essential for glucose oxidation in brain, diffuses more easily from chopped brain than from brain slices.

T. F. Dixon and Meyer (1936) working with ox brain have found that the respiration of minced cerebral cortex in presence of glucose is about half that of the sliced tissue. The respiration of other parts of the brain (cornu ammonis, globus pallidus, thalamus, cerebellar cortex) in presence of glucose is very greatly decreased, on mincing, from the values obtained with intact slices. Clearly various parts of the central nervous system have different susceptibilities to mechanical injury so far as total respiration is concerned. This seems to be particularly the case with the larger cells of the gasserian ganglion. T. F. Dixon and Meyer point out that this fact invalidates the conclusion of Holmes (1932) who, using chopped tissue, found that the respiration of the gasserian ganglion is very low in a glucose medium, this being actually less than that of the cranial nerve. Holmes had suggested on the basis of this result that the seat of metabolic activity may be in the dendrite rather than in the cell body, since the gasserian ganglion cells have no dendritic structures.

It is quite conceivable that the failure of other workers to note large differences between the respirations of sliced and minced tissues has been due to their working with the whole brains of small animals; possibly less mechanical injury on the brain as a whole is effected in

[4] It should be pointed out that, according to Cohen and Gerard (1937), a colloidal aqueous extract of brain can still accomplish various oxidations, but not that of glucose.

these cases. Weil-Malherbe (1937) has recently drawn attention to the fact that the extent of succinic acid formation, during pyruvic acid metabolism, depends greatly on whether sliced or minced tissue is used, and Jowett (1938) has shown that the respiration of sliced brain is more sensitive to a narcotic such as ethyl urethane than was found by Bülow and Holmes (1932) who used minced brain.

TABLE 2

BRAIN CORTEX SLICES	Q_{O_2}	MEDIUM	REFERENCE
Rat	10.7	Ringer-glucose	Warburg (1924)
Rat	14.5	Ringer-glucose	Loebel (1925)
Rat	12.4 (13.7–11.4)	Ringer-glucose	Dickens and Šimer (1930 a, b)
Rat	19.1*	Phosphate-saline-glucose	Jowett and Quastel (1937 c)
Guinea pig	14–15	Phosphate-saline-glucose	Quastel and Wheatley (1934)
Guinea pig	11.8 (over 2 hr. period)	Phosphate-saline-glucose	Jowett and Quastel (1937 b)
Guinea pig	2.7	Ringer only	Wohlgemuth (1933)
Rabbit	6.0	Ringer only	Holmes (1930)
Rabbit	7.3–10.4	Rabbit serum	Krebs and Rosenhagen (1931)
Rabbit	8.3	Ringer-glucose	Dixon (1936)
MINCED BRAIN TISSUE			
Rat	5.3	Phosphate-saline	Quastel and Wheatley (1932)
Guinea pig	3.8		Szent-Gyorgyi (1931)
Rabbit	3.8	Bicarbonate-Ringer	Ashford and Holmes (1931)
Pigeon	3.3	Phosphate-Ringer	Peters and Sinclair (1933)
Guinea pig	3.3–4.6	Phosphate-saline	Quastel and Wheatley (1932)
Guinea pig	6.7	Phosphate-saline-glucose	Quastel and Wheatley (1934)

* Estimated over the first 24 minutes of the experiment. The respiration of rat brain cortex slices falls with time.

Rate of oxygen uptake by brain tissue. Typical results showing the magnitude of the respiration of isolated brain tissue are given in table 2.

It will be noted *a*, that the Q_{O_2} of brain cortex slices is decidedly higher in a glucose medium than in a glucose free medium.

b, that the Q_{O_2} of minced whole brain is lower than that of cortex

slices. This is partly due to the fact that whole brain contains white matter which respires at a lower rate than gray matter. Results with minced whole brain, in absence of added glucose, are also variable because the amount of glucose in the brain when removed from the animal is a variable quantity.

T. F. Dixon and Meyer (1936) have examined the respirations of slices of various parts of ox brain in presence of glucose and give the following results:

	O_2 uptake in µl. per gram wet weight per hour
Cerebral cortex	1,700
Cerebellar cortex	2,550
Corpus striatum	1,980
Cornu ammonis	1,260
Thalamus	1,170
Globus pallidus	360

There is little doubt that the Q_{O_2} of brain cortex slices examined under the most favorable conditions so far is lower than that expected from the values of oxygen consumption of brain *in vivo*. Conceivably the latter are too high, but it is more likely that the optimal conditions for the examination of brain tissue *in vitro* have not yet been secured. The respiration of brain cortex slices in a glucose medium varies with time; it falls off much more with time in the case of rat than in the case of guinea pig, and the rate of fall depends very greatly on the ionic concentration of the medium. With rat cortex a Q_{O_2} of 19.1 was found during the first 24 minutes of the experiment; a higher figure would obviously have been obtained had it been possible to make an accurate estimation during the first few minutes of examination of the tissue. Even this figure is not remote from the values calculated from the *in vivo* studies.

Brookens, Ectors and Gerard (1936) have examined the respiration of plugs of cerebral cortex with a capillary respirometer. They have found values of $Q_{O_2} = 10$, but with a technique permitting readings within the first few minutes a linear extrapolation to zero time gave values of $Q_{O_2} = 25$.

Respiratory quotient of brain in vitro. Loebel (1925) showed that the R. Q. of brain slices in a fructose medium was 0.99. Dickens and Šimer (1930b, 1931) found that the R. Q. of normal rat brain cortex slices in a glucose medium is 0.99.

Wortis (1935) investigated the respiratory activity of minced brain tissue of cat and rat and found under normal conditions an R. Q. of unity. Himwich and Fazekas (1932) have determined the respiratory quotients for various parts of rat brain and have obtained the following results: cortex, 0.99; brain stem, 0.93; cerebellum, 0.89; medulla, 0.89. Dickens and Greville (1933) working with rat and rabbit brain cortex slices obtained an R. Q. of unity which was maintained even in the absence of added glucose to the medium. Elliott and Baker (1935) reported a lower value for the R. Q. of rat brain cortex (0.93–0.78) but Dickens (1936) points out possible inaccuracies in their experiments, and shows that the R. Q. of rat brain cortex in glucose, fructose or lactate media is unity.

It is evident that the respiratory quotient of normal brain examined *in vitro* is approximately unity, a value which agrees with the determinations on brain *in vivo*. Presumably carbohydrate oxidation is responsible for this quotient. This conclusion is supported by the work of K. C. Dixon (1936). This worker showed that the amount of glucose disappearing aerobically in the presence of brain slices is equal to that expected from the oxygen consumption (assuming complete oxidation) and from the amounts of lactic acid formed by the aerobic glycolysis. His figures, using rabbit brain cortex, are as follows:

Q_{O_2} = μl O_2 absorbed per mgm. dry wt. tissue per hr. = 8.3

∴ Glucose oxidised to account for this consumption of O_2, $= \frac{8.3}{6}\,\mu l = 1.38\,\mu l$

since 1 mol. glucose requires 6 mols O_2 for complete oxidation.

$Q_M^{O_2}$ = aerobic glycolysis = μl lactic acid formed aerobically per mgm. dry wt. tissue per hr. = 2.8 μl

∴ Glucose broken down to account for this amount of glycolysis $= \frac{2.8}{2} =$ 1.4 μl

since 1 mol. glucose produces 2 mols lactic acid.

∴ Total glucose broken down = 1.38 + 1.4 = 2.78 μl per mgm. dry wt. tissue per hour.

Amount of glucose found to be broken down aerobically = 2.4 μl; 2.6 μl; 3.5 μl; 3.2 μl; 4.0 μl (average = 3.14 μl) per mgm. dry wt. tissue per hour.

The correspondence is reasonably good, and indicates that the respiration of brain cortex *in vitro* can be mainly accounted for on the basis of oxidation of glucose.

Effects of change of temperature on brain respiration. K. C. Dixon

(1936) has studied the effects of abnormally high temperatures on the metabolism of cerebral cortex. The following results may be quoted:

	Q_{O_2}			
37°	6.8,	8.7,	8.1,	9.0
42°	8.0,	8.2,	9.4	
45°	17.0,	13.0,	16.8,	16.8.

These results were obtained using rabbit brain cortex in a bicarbonate-Ringer solution containing glucose. At 42° the rate of respiration was only slightly increased above that at 37°, but at 45° the respiration was increased very greatly above that at 37°. Similarly the amount of glucose broken down is greatly increased at 45° over that at 37°.

Dixon cites the following results for the change in respiration and aerobic glycolysis for the temperature range 42–45°.

TEMPERATURE	Q_{O_2}	$Q_M^{O_2}$ (AEROBIC GLYCOLYSIS)
°C.		
42	8.4	2.7
43	10.8	3.4
44	11.3	5.5
45	11.0	11.3

It seems to be clear that above 42° there is a definite change in the equilibria governing glucose breakdown in the brain.

Comparison of respirations of gray and of white matter. The respiratory activity of white matter is about one-third to one-quarter that of of gray matter (cf. Winterstein, 1922). Holmes (1930) gives 1200 μl per gram per hour for gray matter of rabbit brain and 300 μl (Q_{O_2} = 1.5) per gram per hour for the white matter.

Brookens *et al.* (1936) using a capillary micro-respirometer state that respiration values for various portions of white matter range from 650 to 1800 μl O_2 per gram per hour and for gray matter from 800–2500 μl per gram per hour.

Krebs and Rosenhagen (1931) give a range of 2.8–4.6 as the Q_{O_2} for rabbit brain white matter while the Q_{O_2} of gray matter of basal ganglia is given as 7.6–9.9.

Effects of diffusible metabolites on brain respiration. Washed brain tissue (in absence of added metabolites) respires less than fresh unwashed tissue. Holmes (1930) showed that the respiration of mouse brain falls very greatly after the tissue is washed. Peters and Sinclair

(1933) found that the respiration of pigeon brain fell rapidly when the minced tissue was immersed in a Ringer-phosphate medium. They thought this was due to a gradual inactivation of an essential enzyme system, until further study made it evident that the addition of various metabolites (lactate, pyrophosphate and α-glycerophosphate) had a considerable influence on the respiratory rate. Quastel and Wheatley (1932) found that fresh minced guinea pig brain tissue takes up oxygen at a rate of 200 μl per gram per 15 minutes ($Q_{O_2} = 4$) for about two hours after which the respiratory rate begins appreciably to fall reaching about half the initial rate after three hours. On addition of glucose or sodium lactate to the tissue at this stage the rate of oxygen uptake immediately increases. Moreover the addition of glucose to the brain tissue at the commencement of the experiment leads to a much longer maintenance of the Q_{O_2} at the initial level.

There can be little doubt that diffusible metabolites are largely responsible for the maintenance of respiration in brain. The rapid fall in respiration which brain tissue exhibits, in a metabolite free medium, is due partly to depletion of metabolites in the tissue, either by oxidation or by diffusion into the external medium so that their effective concentrations are lowered.

Action of cyanide on brain respiration. Cyanide has a large inhibitory action on brain respiration, but according to M. Dixon and Elliott (1929) and to Banga, Schneider and Szent-Gyorgyi (1931) an appreciable percentage of the respiration is not inhibited by cyanide. It must be borne in mind however that cyanide inhibition depends very much on the experimental conditions (Alt, 1930). Torrès (1935) finds that 97.5 per cent of the respiration of rat brain examined in Ringer solution is inhibited by $\frac{N}{200}$ cyanide; the Q_{O_2} in presence of cyanide being 0.27. According to Himwich *et al.* (1933) cyanide while diminishing the oxygen uptake of brain cortex does not affect the respiratory quotient.

The pathological changes which take place in brain after the administration of cyanide to animals have been investigated by Meyer (1933).

Marshall and Rosenfeld (1937) have studied the action of small doses of cyanide as a respiratory stimulant and have shown that respiratory stimulation from the cyanide may occur in dogs and cats with no decrease in the oxygen consumption of the whole animal. They point out that stimulation from cyanide resembles very closely that from anoxemia and explain this "by assuming that the sino-aortic receptors

are more readily affected than other tissues." It is well known that different tissues vary in their sensitivity to cyanide *in vitro* (cf. Kisch, 1933).

Chang and Gerard (1931) have shown that cyanide largely abolishes the resting oxygen consumption of vertebrate and invertebrate nerve and that the cyanide inhibition can be reversed by the addition of a reducible dyestuff such as cresyl blue.

The large inhibitory effect of cyanide on brain respiration points to the probability that the respiration proceeds largely through the cytochrome-cytochrome oxidase system. The small cyanide-insensitive part of the respiration may be due to the activity of a flavine system.

Action of carbon monoxide. Little is known of the action of CO on brain oxidation but Chang and Gerard (1931) have recorded its effects on nerve respiration. In 98 per cent CO + 2 per cent O_2 the resting respiration of frog nerve, in presence of cresyl blue, is depressed 70 per cent in the dark and less than 5 per cent in the light. Schmitt (1930) discusses the inhibition of nerve respiration by CO and the action of the light. The action of CO on the metabolism of retina, among other tissues, is described by Laser (1937).

The fact that intact nerve in presence of CO does not respond to the action of a dye to the extent that nerve in presence of cyanide responds makes it likely that the actions of CO and of cyanide on nerve respiration are not identical. The reader is referred, however, to Gerard's monograph (1932) for details concerning nerve metabolism.

The pathological changes found in brain following carbon monoxide have been described by Meyer (1932).

Indophenol (cytochrome) oxidase of brain. Indophenol oxidase activity of brain was studied by Vernon (1911) who showed that the intensity of the reaction varied inversely with the size of the animal. The addition of *p*-phenylenediamine to brain tissue greatly increases its oxygen uptake. Holmes (1930) finds that the ratio of oxygen uptakes in presence of *p*-phenylenediamine with various parts of cat brain is as follows: gray matter: white matter: nerve = 26.2:4.3:1. Quastel and Wheatley (1932) have examined the activities of brain tissue of various animals towards *p*-phenylenediamine and their results are shown in the accompanying table (table 3) taken from their paper.

Fresh brain tissue was used in the experiments referred to in table 3 (with the exception of man). The relatively high value of the autoxidation of these tissues makes it difficult to estimate precisely the amount of oxidation of *p*-phenylenediamine, for there may be competition be-

tween the diamine and the substrates responsible for the autoxidation. Clearer results were obtained with tissues which had been allowed to deplete themselves considerably of their substrates by respiration in a

TABLE 3

O_2 uptake in µl. (at 37°) by 0.5 gram minced whole brain tissue alone and in the presence of 21 mgm. p-phenylenediamine hydrochloride (neutralised) in 90 minutes

Theoretical O_2 uptake for combination of one atom O_2 with this quantity of p-phenylene diamine = 1474 µl.

	ALONE	WITH *p*-PHENYLENEDIAMINE	EXTRA O_2 UPTAKE
Mouse	696	2,212	1,516
Rat	680	1,428	748
Guinea pig	645	1,480	835
Rabbit	685	1,525	840
Pigeon	844	2,310	1,466
Cat (grey matter)	422	1,560	1,138
Man (grey matter, from brain twelve hours after death)	444	1,402	958

phosphate medium for 3 hours at 37° before the addition of the diamine. The following results were then obtained (conditions as in table 3):

	ALONE	O_2 UPTAKE µL. IN 1 HOUR WITH *p*-PHENYLENEDIAMINE	EXTRA O_2 UPTAKE
Rabbit	162	1315	1153
Mouse	154	1534	1380
Guinea pig	154	1406	1252

The rates of oxygen uptake in presence of *p*-phenylenediamine (i.e., the relative indophenol (or cytochrome) oxidase activities) were inversely proportional to the size of the animal. Considering the wide difference between the indophenol oxidase activities of gray and of white matter, it is possible that the inverse relationship noted is due to varying proportions of gray to white matter in the brains of these animals, the smaller animals having the greater proportion of gray matter.

Quastel and Wheatley (1932) found the Q_{O_2} of brain in presence of *p*-phenylenediamine to rise rapidly from a value of 4 to a value of about 17 and then to fall rapidly as the diamine became exhausted within the next 90 minutes to a Q_{O_2} of about 2. There is evidence that the products

of oxidation of the diamine inhibit the oxidation by brain of its normal substrates.

These facts make it clear that brain in common with other mammalian tissues possesses an enzyme for the activation of molecular oxygen. The strong inhibitive action of cyanide on normal respiration of brain points to the fact that the indophenol (cytochrome) oxidase is quantitatively the most important mechanism for oxygen activation.

So far as other cyanide sensitive enzymes are concerned, peroxidase exists to a small extent in brain and so does catalase, though the amount of the latter is slight, perhaps one hundredth of the amount in liver (Schmitt and Skow, 1933; Elliott and Bancroft, 1934).

TABLE 4

Q_{O_2} of minced guinea-pig brain which had been allowed to respire for 3 hours in phosphate saline medium prior to the addition of sugars

The initial Q_{O_2} was 4.0

SUGAR (0.06 PER CENT)	TIME FROM ADDITION OF THE SUGAR	
	30 minutes	120 minutes
None (control)	1.7	1.1
Glucose	2.9	4.0
Fructose	3.5	3.5
Mannose (0.025%)	2.8	2.9
Galactose	2.5	1.7
Mannitol	1.9	1.2
Xylose	1.5	1.3
Arabinose	1.3	1.0
Glycogen	1.8	1.7

Cytochrome. Cytochrome exists in brain but little is known about its distribution in this organ. According to Holmes (1932) there is about half as much cytochrome in cerebral cortex as in yeast, more than in skeletal muscle but less than in heart muscle. White matter from the cord contains one-quarter to one-fifth as much as cortex and it cannot be stated with certainty whether any cytochrome exists in nerves.

Oxidation of carbohydrates by brain in vitro. In confirmation of the known facts concerning R. Q. of brain and glucose removed from the blood in its passage through the brain, the addition of glucose to brain tissue *in vitro* greatly prolongs the steady rate of oxygen uptake. Fructose and mannose also increase the oxygen uptake of brain tissue (Loebel.

1925). The work of Dickens and Simer (1931), Ashford and Holmes (1931), Quastel and Wheatley (1932) indicates the importance of glucose oxidation to brain *in vitro*. The last workers studied the effects of various sugars on the oxidations by brain tissue which had been allowed to deplete itself largely of oxidizable material, prior to the addition of the sugar, by autoxidation for three hours. Typical results are shown in table 4. Of all the sugars tested, glucose, fructose and mannose have the largest influence in restoring the Q_{O_2} to a rate approaching the initial value. Galactose has a small effect, but mannitol, arabinose and xylose are practically inert at the concentrations tried (see also Sherif and Holmes, 1930).

The relative rates of oxidation of glucose by minced brain tissues from various animals are shown by the following figures: (the brain tissue had been allowed to respire for 3 hours prior to the addition of the glucose). The figures refer to μl O_2 uptake in 1 hour at 37° by 0.5 gram brain tissue.

ANIMAL	NO GLUCOSE ADDED	GLUCOSE (0.025 PER CENT) ADDED
Rabbit	162	229
Guinea pig	154	287
Mouse	154	345

It will be observed that, as in the case of indophenol oxidase activity, the smaller the animal the greater the rate of oxidation of glucose by the brain.

With minced pigeon brain (cerebrum) Gavrilescu *et al.* (1932) obtained the following figures.

MEDIUM	O_2 UPTAKE μL. PER GRAM PER HOUR
Ringer	775; 805
Ringer + glucose 0.25 per cent	1645; 1955

The respiration of brain cortex slices falls quickly with time at 37° in a phosphate-Ringer medium in the absence of glucose. With glucose present the rate remains approximately constant for at least two hours. The values given in table 5 illustrate this fact; they refer to guinea-pig brain cortex.

TABLE 5

MEDIUM	Q_{O_2} TIME FROM COMMENCEMENT OF THE EXPERIMENT				REFERENCE
	30 minutes	60 minutes	90 minutes	120 minutes	
Phosphate-glucose 0.16%......	15.8	16.2	16.7	14.3	Quastel and Wheatley (1934)
Bicarbonate-glucose 0.2%.....		10.4		9.1	Dickens (1936)

With rat brain cortex in a phosphate medium the Q_{O_2} falls more rapidly than with guinea-pig brain. Typical values (Jowett and Quastel, 1937) for the respiration of rat brain cortex in the absence and in the presence of glucose and in a phosphate-saline medium are as follows:

Q_{O_2} *(mean value for a 2 hour period)*

No glucose added....................................	2.89 ± 0.18
With glucose 0.01 M..................................	12.20 ± 0.33

Dickens and Greville (1933) give the following figures as illustrative of the respiration of rabbit and rat brain cortex in Ringer media with and without carbohydrate

	Q_{O_2}	
	Rat	Rabbit
No carbohydrate added..................	3.9	5.0
Glucose present........................	12.8	7.5
Fructose present.......................	9.7	8.5

Holmes (1932) showed that the respiration of the brain of an animal killed after it had been allowed to convulse after administration of insulin was less than that of a normal animal—the brain tissues being examined in glucose free media. This is due to the fact that the respiration of brain in a glucose-free medium depends upon its store of metabolites, of which glucose (or lactic acid) is the most important. The amount of this in the brain is lessened when the blood sugar falls after insulin administration. Wortis (1935) confirmed this fact, using minced brain, and gave the figures shown in table 5a (see also Wortis, 1938).

His results indicate that the brain of an insulin treated animal respires normally in a glucose medium;[5] the fall in respiratory activity as well as the drop in R. Q. in a glucose-free medium shows that the brain of such an animal must contain very little glucose. These observations are also confirmed by the work of Himwich and Fazekas (1937), who have shown that the difference between the glucose contents of arterial blood and cerebral venous blood of dogs under intense hypoglycemia is only 3 mgm. per cent, as compared with a normal difference of about 13 mgm. per cent. Moreover the oxygen consumption of dog brain *in vivo* falls from a normal figure of 7.99 volumes per cent to 3.80 volumes per cent during intense hypoglycemia.

TABLE 5a

	R.Q.		Q_{O_2} (60 minutes)	
	Ringer-phosphate	Ringer-phosphate-glucose 0.2%	Ringer-phosphate	Ringer-phosphate-glucose 0.2%
Normal rat brain	1.00	1.01	3.6	5.5
Brain of rat injected with insulin until convulsions occurred	0.73	0.92	1.4	5.2

There can be little doubt that during the hypoglycemia induced by insulin there is a lessened consumption of oxygen by brain and lessened glucose utilization by this organ. The intravenous injection of glucose to a hypoglycemic animal restores the oxygen consumption by the brain *in vivo* to the normal level.

Dameshek, Myerson and Stephenson (1935) compared the contents of glucose and of oxygen in the vessels supplying the human brain before and after intravenous injection of insulin. They took samples of blood from the brachial artery and the internal jugular vein and came to the conclusions that the uptake of glucose by the brain is materially reduced during a severe hypoglycemic reaction and that the uptake of oxygen by the brain varies indirectly with the severity of the insulin reaction, becoming much reduced during the most severe reactions.

Himwich, Bowman, Wortis and Fazekas (1938b) have confirmed these findings. They have examined the oxygen content of blood from the internal jugular vein and femoral artery in schizophrenic patients and the same patients treated with insulin, in the hypoglycemic "shock"

[5] See also Gerard and Schachter (1932).

therapy. They state that before insulin is injected the average oxygen utilization is 7.04 vols. per cent, and that of glucose 12.5 mgm. per cent. After insulin injection, whilst the patients are in the pre-comatose condition, the oxygen consumption falls to 6.1 vols. per cent and the utilization of glucose falls to 7 mgm. per cent. It is evident that insulin, in lowering the blood glucose, diminishes access to the entire central nervous system of its most important substrate for respiratory purposes.[6] Probably the various neurological disturbances which manifest themselves during hypoglycemia are due to the resulting anoxemia in the nervous system although oxygen is freely available as shown by the relatively high oxygen content of the cerebral venous blood.

The brain tissue of depancreatized or diabetic animals burns lactic acid and glucose (Holmes and Holmes, 1927; Baker, Fazekas and Himwich, 1938) at the normal rates. Table 6 shows results given by the latter workers.

TABLE 6

CEREBRAL CORTEX OF	GLUCOSE (0.02M)		LACTATE (0.02M)	
	Q_{O_2}	R.Q.	Q_{O_2}	R.Q.
Normal cat	9.1	0.95	7.8	1.01
	8.7	0.94	10.7	0.88
	7.1	0.99	8.7	0.97
Diabetic cat	8.8	0.97	9.7	0.86
	8.9	1.01	9.1	0.90
	10.2	0.98	9.6	1.03

It is evident that insulin lack does not affect the ability of brain to burn glucose. The addition of insulin to a glucose-Ringer medium has no effect on the glucose oxidation by brain (Wortis, 1938).

Carbohydrate breakdown in brain. Glucose is not converted into glycogen by brain (Holmes and Ashford, 1930) and apparently glycogen[7] is not stored by brain (Takahashi and Asher, 1925). It is evident, however, from the results of Kerr and Ghantus (1936) and Kerr (1938) that glycogen is definitely present in the brain. The results of Gaddie and Stewart (1935) and Geiger (1935) make it clear that the glycogen breakdown in brain is small compared with that in muscle.

[6] Kerr, Hampel and Ghantus (1937), and Kerr and Ghantus (1936), have pointed out that insulin administration decreases the levels of both glycogen and free sugar in the brain of normal cats.

[7] For a short review of glycogen metabolism in brain see Page, *Chemistry of the Brain*, 1938, p. 183.

Holmes (1930) and Krebs (1931) have given reasons for the belief that glucose oxidation in brain proceeds through lactic acid. This conclusion, however, can no longer be held owing to the recent finding that lactate oxidation in presence of brain can be inhibited to a greater extent than glucose oxidation. This finding was made independently by Jowett and Quastel (1937a), and by Baker, Fazekas and Himwich (1938). (See also Himwich and Fazekas, 1935.) The former used as

TABLE 7

ANIMAL	INHIBITOR	Q_{O_2} SUBSTRATE		PER CENT EFFECT OF INHIBITOR ON Q_{O_2}		REFERENCE
		Glucose 0.02M	*dl* lactate 0.02M	Glucose	*dl* lactate	
Guinea pig ..	Nil	15.8	11.1*			Jowett and Quastel (1937 a)
	Sodium hydroxy-malonate 0.07M	11.1	6.6*	−30	−52	
Rat...	Nil	13.8	13.1*			Jowett and Quastel (1937 a)
	Sodium hydroxy-malonate 0.07M	12.5	9.2*	−9	−30	
Rat....	Nil	15.0	15.0			Baker, Fazekas and Himwich (1938)
	Nicotine 0.03M	8.3	1.8	−45	−88	
Cat....	Nil	9.0	9.4			Baker, Fazekas and Himwich (1938)
	Nicotine 0.03M	6.5	1.3	−28	−86	
Rat....	Nil	13.5	13.5			Baker, Fazekas and Himwich (1938)
	Nicotinic acid 0.03M	12.5	4.2	−7	−69	

* = Lactate 0.01M

inhibitor sodium hydroxymalonate which is known to be a specific inhibitor of lactic dehydrogenase (Quastel and Wooldridge, 1928); the latter used nicotine whose mechanism of action on enzymic reactions is as yet unknown. Relevant figures showing the actions of these inhibitors on lactate and glucose oxidation by brain cortex are given in table 7.

Glucose cannot be directly oxidized to CO_2 and water, i.e., with-

out the formation of intermediate substances, for the addition of glucose to brain tissue already burning lactate does not increase the rate of oxygen uptake (Quastel and Wheatley, 1932), a fact confirmed by Elliott, Greig and Benoy (1937). It was thought originally that this supported the contention that glucose oxidation proceeds *via* lactic acid, but in view of what has already been stated, another explanation must be sought for the lack of additive action. Such an additive action should take place if the oxidations of glucose and of lactic acid were quite independent. It is improbable that lactate oxidation proceeds through glucose as an intermediary because *a*, concentrations of fluoride and iodoacetate which inhibit glucose oxidation do not inhibit lactate oxidation (Holmes, 1930; Krebs, 1931; Quastel and Wheatley, 1932); *b*, brain slices fail to synthesize glucose from lactate under conditions where synthesis takes place with liver and kidney slices (Benoy and Elliott 1937). The most obvious explanation of the fact that glucose and lactate oxidations show no additive effects is that both substances proceed through a common intermediate, whose oxidation accounts for most of the oxygen uptake. Such an intermediate might be pyruvic acid, thus:

$$\begin{array}{lll} \text{Glucose} \searrow & & \\ & \text{Pyruvic acid} \longrightarrow & \text{Final oxidation products} \\ \text{Lactic acid} \nearrow & & \end{array}$$

According to Elliott *et al.* (1937) the addition of glucose does not increase the rate of respiration of rat brain cortex in presence of pyruvate.

It has long been known that fructose can be oxidized by brain at a rate comparable with that of glucose, although little breakdown of fructose to lactic acid takes place in presence of brain (Loebel 1925). Haarman (1932) gives the ratio of glycolytic activities of glucose, fructose, hexosediphosphate and glycogen in presence of brain tissue (rabbit, dog) as 25.4: 3.7: 3.2: 1.0. Dickens and Greville (1933) conclude from their experiments that fructose is directly oxidized by brain cortex. Iodoacetate inhibits fructose oxidation by brain (Quastel and Wheatley, 1932) and it is conceivable that fructose is oxidized by a path similar to that of glucose.

Oxidation of lactic acid in the brain. Lactic acid arises from glucose in presence of brain, both aerobically and anaerobically, the rate of anaerobic formation being far greater than that aerobically. A dis-

cussion, however, of glycolysis in brain and of the action thereon of oxygen, is outside the scope of this review.

Lactic acid is a normal constituent of blood and must be considered, therefore, as a factor contributing to the respiration of brain: Himwich *et al.* (1938) state that it is doubtful whether lactic acid is utilized by the brain to any appreciable extent *in vivo* and give figures which make it seem unlikely that lactic acid is removed from the blood on its passage through the human brain. It seems, however, that their experiments are by no means conclusive on this point, and the question of whether lactic acid is oxidized by brain *in vivo* must be left in abeyance until further work has been done. Lactic acid, like glucose, is stated to be absorbed from the blood by the brain of normal dogs (McGinty, 1929) and by the brain of diabetic dogs (Himwich and Nahum, 1929).

TABLE 8

PER CENT CONCENTRATION OF LITHIUM LACTATE ADDED	μL O_2 EQUIVALENT TO LACTIC ACID DISAPPEARANCE	TOTAL μL O_2 TAKEN UP	RATIO
0.025	2350	4710	0.5
0.05	3000	4875	0.61
0.1	4450	5000	0.89
0.2	6750	6450	1.05
0.3	9200	6700	1.37
0.4	8300	6480	1.28
0.5	8260	6300	1.30
0.6	10000	7100	1.40
0.8	10500	7000	1.44

There is no doubt that lactic acid prolongs the oxygen uptake of brain (Loebel, 1925; Meyerhof and Lohmann, 1926; Ashford and Holmes, 1929). The last authors made a careful examination of the disappearance of lactic acid acid aerobically in brain and have shown that more lactic acid disappears than can be accounted for by the extra oxygen uptake due to the added lactate. No satisfactory explanation of this phenomenon is as yet forthcoming. An obvious explanation is that the lactic acid exercises a "sparing" action on the oxidation of other metabolites, i.e., that the "extra O_2" is not a true indication of the amount of lactic acid oxidized. Moreover such a sparing action can be experimentally demonstrated. But in certain of the experiments of Ashford and Holmes the *total* oxygen uptake of the brain is less than that expected for the complete oxidation of the lactic acid which has disappeared. Table 8, taken from their paper, illustrates this.

It is evident from these data that with concentrations of lithium lactate above 0.2 per cent, the discrepancy between O_2 uptake and the lactic acid removed cannot be explained entirely by the sparing action of lactate. At the lower concentrations, however, which are more biologically significant, a sparing effect would be an ample explanation.

Quastel and Wheatley (1932) have shown that the addition of lactate and pyruvate to brain tissue, partially depleted of its metabolites by autoxidation for 3 hours, restores the rate of respiration practically to that found with the fresh tissue. The rate of oxidation secured by lactate (*dl*) is almost identical with that obtained by an equivalent concentration of glucose; this fact affords additional evidence for the view that the oxidations of glucose and of lactic acid proceed through the intermediate formation of a common step—possibly pyruvic acid. These authors also showed that the presence of *dl* lactate exerts a "sparing" effect on the oxidation of sodium succinate by brain tissue.

Peters and Sinclair (1933) observed that pyrophosphate increases the rate of oxidation of lactate by minced pigeon brain; the effect is enhanced by the addition of magnesium ions. According to these authors one-third of the respiration of pigeon brain tissue in presence of lactate is inhibited by fluoride. They found that the addition of pyruvate to lactate caused a slight increase in respiration of minced pigeon brain.

The fact that biological oxidation of lactic acid leads to the formation of pyruvic acid is well known. The rapid rate of pyruvic acid oxidation by brain tissue has made it difficult to detect pyruvic acid formation when lactic acid is being burned by brain. That it is formed under such circumstances, however, has been shown in the case of minced pigeon brain by Peters and Thompson (1934) and in the case of rat and guinea-pig brain cortex slices by Jowett and Quastel (1937b). The amounts of pyruvic acid found are, as might be expected, small; the concentration found in a lactate medium in which brain tissue is respiring is presumably in dynamic equilibrium with that in the tissue slices. The pyruvate concentration found with guinea-pig or rat brain cortex slices in a 0.02 M *d* lactate medium is of the order 2×10^{-4} M.

The amount of pyruvate found in a lactate medium in which pigeon brain tissue is respiring is, according to Peters and Thompson (1934) markedly increased, if iodoacetate (0.0005 M) is also added to the medium. Presumably the iodoacetate inhibits the breakdown of pyruvate. Further work showing that lactic acid oxidation in pigeon brain proceeds through pyruvic acid has been carried out by Peters (1936).

As has already been pointed out hydroxymalonate which is an inhibitor of lactic dehydrogenase inhibits the oxidation of lactic acid by brain (table 7). Lactic dehydrogenase, as well as other dehydrogenases, exists in brain tissue, their presence having been demonstrated by the anaerobic methylene blue technique.

Herter in 1905 showed that methylene blue is reduced by brain *in vivo*, the reduced dye being reoxidized on exposure to air. Thunberg and Sherif have shown nerve tissue to possess dehydrogenase systems.

Holmes (1930) using the methylene blue technique gives the figures shown in table 9 illustrating the activity of ox brain, and pointing clearly

TABLE 9

	REDUCTION TIME	
	Grey matter	White matter
	minutes	*minutes*
Control (no added substrate)	13½	70
M/50 Lithium lactate	7	24
M/100 Glucose	6	48
M/100 Galactose	10	70

TABLE 10

SUBSTRATE	M CONCENTRATION AT WHICH HALF THE MAXIMUM VELOCITY OF REDUCTION IS REACHED
Glucose	0.0135
Fructose	0.021
Sodium lactate	0.0045
Sodium pyruvate	0.0052
Sodium citrate	0.018
Sodium succinate	0.004

to the existence of lactic dehydrogenase in brain. The amount of the dehydrogenase is greater in gray than in white matter.

Ashford and Holmes (1931) state that lactate and glucose have approximately the same activities as hydrogen donators to methylene blue. Davies and Quastel (1932) find, however, differences between these activities as shown in table 10 taken from their paper. This table gives the Michaelis constants of various hydrogen donators in presence of minced ox brain tissue.

A study of the lactic dehydrogenase system of brain and of other

organs has been carried out by Green and Brosteaux (1936). It is now well known that a necessary part of the lactic dehydrogenase system in mammalian tissue is cozymase and it has been shown recently by Quastel and Wheatley (1938) using their ferricyanide technique, that the presence of cozymase markedly increases the oxidation of *dl* lactate (by ferricyanide) in rat brain cortex slices. Since the system lactate-lactic dehydrogenase-cozymase reduces ferricyanide but not methylene blue, and since brain tissue in presence of lactate reduces both ferricyanide and methylene blue, it follows that brain contains a factor which transfers hydrogen from reduced cozymase to methylene blue. Evidence for such a carrier has now been obtained by Euler and Hellstrom (1938) and by Dewan and Green (1938).

That cozymase exists in brain has been shown by Sym, Nilsson and Euler (1930) who give the values shown in table 11.

TABLE 11

	COZYMASE UNITS
Brain grey matter	3.7
Brain white matter	1.0
Kidney cortex	5.2
Liver	3.2
Blood	0.1

Oxidation of pyruvic acid by brain. A number of workers has shown that pyruvate is highly effective in maintaining the respiration of brain, either minced or in the form of slices (e.g. Loebel, 1925; Ashford and Holmes, 1931; Quastel and Wheatley, 1932; Peters 1936; Lipmann 1937; Elliott, Greig and Benoy, 1937). With rat brain cortex there is little difference between the Q_{O_2} in presence of glucose 0.01 M and the Q_{O_2} in presence of pyruvate 0.02 M; if anything the respiration may be a little less in pyruvate. The following figures may be cited as typical (Jowett and Quastel, 1937b).

Substrate	Q_{O_2}
None added	2.89 ± 0.18
Glucose 0.01 M	12.20 ± 0.33
Pyruvate 0.02 M	11.10 ± 0.65

It has been known for some time that pyruvate increases the respiration of muscle, liver and kidney in a similar manner to lactate (Meyerhof 1930; Dickens and Šimer 1930b).

That pyruvic acid disappears when brain tissue respires in presence

of it is also now well known (Peters and Thompson, 1934, etc.). The rate of aerobic disappearance is given in table 12.

The disappearance of pyruvic acid is greater than can be accounted for by the oxygen uptake, assuming that complete oxidation of pyruvic acid takes place. This is seen in the results of Jowett and Quastel (1937b) and of McGowan (1937), the former working with rat brain and the latter with pigeon brain. The inference would be that pyruvic acid undergoes a secondary change in presence of brain tissue. A part of this change consists of reduction to lactic acid. Haarman (1932) showed that brain tissue reduces pyruvate to lactate under anaerobic conditions. Elliott *et al.* (1937) found that in a mixture of glucose and pyruvate, in presence of rat brain cortex examined aerobically, most of the pyruvate disappearance can be accounted for by lactate formation. It is evident that this is not the case in the absence of glucose. Krebs

TABLE 12

BRAIN CORTEX OF	PYRUVATE CONCENTRATION	Q_p (RATE OF DISAPPEARANCE OF PYRUVIC ACID) IN μL PER MGM. DRY WEIGHT TISSUE PER HOUR	REFERENCE
Rat.........	0.04M	4.3; 6.2	Elliott, Greig, and Benoy (1937)
Pigeon......	0.005M	4.3	Lipmann (1937)
Rat.........	0.01M	7.6; 8.4	Jowett and Quastel (1937 b)
Rat.........		9.3; 8.3	Krebs and Johnson (1937 a)

and Johnson (1937) have shown that anaerobically two molecules of pyruvic acid undergo a Cannizzaro reaction in presence of brain tissue to form acetic, lactic and carbonic acids in equimolecular quantities. This has been confirmed by Lipmann (1937) and Weil-Malherbe (1937a). The same phenomenon was shown earlier by Nelson and Werkman (1936) to take place in the presence of bacteria (*lactobacillus lycopersici*), confirmed by Krebs (1937) for staphylococci and indeed Quastel and Stephenson some years earlier (1925) suggested that this reaction would account for the anaerobic growth of *B. coli* in pyruvate media. Assuming, however, that the Cannizzaro reaction does take place aerobically in brain tissue, McGowan (1937) comes to the conclusion that the lactic acid formation aerobically does not account for that amount of pyruvate disappearance which cannot be accounted for by the oxygen uptake.[8]

[8] Long, 1938, has very recently shown that lactic and acetic acids are formed when minced pigeon brain respires in a pyruvate medium, the quantities accounting for 30 per cent of the pyruvate disappearing. The rest of the pyruvate seems finally to be completely oxidized.

It seems fairly clear that pyruvic acid may undergo a variety of reactions, and at present intensive research is being carried out in various laboratories to discover the nature of these reactions. It has been shown of late (Kritzmann, 1938; see also Braunstein and Kritzmann, 1937) that in brain tissue pyruvic acid reacts with glutamic acid to form alanine, a transference of the amino group taking place. Weil-Malherbe (1936) showed that brain slices synthesize glutamine from pyruvic acid and ammonia, α-ketoglutaric acid being supposed to be the intermediate in this reaction. He found (1937b) that succinic acid is formed when pyruvic acid or α-ketoglutaric acid is added to brain under anaerobic conditions. Aerobically succinic acid is not found with brain slices or minced brain, presumably owing to the activity of the succinic dehydrogenase present; in presence of malonate, an inhibitor of this enzyme, some succinic acid accumulates with minced brain tissue but not with brain slices. The mechanism by which pyruvic acid is transformed

TABLE 13

SUBSTRATE	Q_{O_2} IN ABSENCE OF INHIBITOR	Q_{O_2} IN PRESENCE OF SODIUM HYDROXYMALONATE 0.07M	PER CENT INHIBITION
dl Lactate 0.01M	14.0	8.8	37
Pyruvate 0.01M	12.6	11.9	5

anaerobically to succinic acid is not clear (Weil-Malherbe, 1937a) and indeed, it seems very doubtful how any of the changes which pyruvic acid is so far known to undergo anaerobically can account for the aerobic oxidation of the ketonic acid. Krebs and Johnson (1937a) believe that the dismutation reaction which pyruvic acid undergoes anaerobically with brain tissue precedes the oxidation process, so that in effect the oxidation of pyruvic acid becomes the oxidation of lactic acid produced by the dismutation. This, however, is unlikely to be the case since Jowett and Quastel (1937a) have shown that hydroxymalonate inhibits *d*-lactate and *dl*-lactate oxidation by rat brain cortex slices to a much greater extent than pyruvate oxidation. This fact is shown in table 13.

The possibility that pyruvic acid is oxidized aerobically through succinic acid has been often considered (Toenniessen and Brinkmann, 1930; Elliott and Schröder 1934; Elliott and Greig, 1937; Weil-Malherbe, 1937b; Krebs, 1937) and undoubtedly the hypothesis is an attractive one. Quastel and Wheatley (1932), however, could not reconcile the hypothesis with the fact that there is no demonstrable formation of

l-malate when brain tissue is respiring in presence of pyruvate. The oxidation of *l*-malate is so slow compared with that of pyruvate by minced brain that some accumulation of *l*-malate should have occurred. These authors showed that with minced brain tissue succinate is oxidized quantitatively to *l*-malate, which can be estimated with fair accuracy by polarimetric means. McGowan and Peters (1937) disagree with the view that succinate is a necessary intermediate in the oxidation of pyruvate.

Whether pyruvic acid oxidation in brain can be adequately interpreted by some such cycle as the "citric acid cycle" of Krebs and Johnson (1937b) has still to be determined and at present it must be concluded that the main mode of aerobic breakdown of pyruvic acid in brain is unknown.

The action of vitamin B_1. A large literature has now accumulated on the action of vitamin B_1 on pyruvic acid metabolism, first discovered by Peters and his colleagues, and it will not be possible to deal with the phenomenon here in any detail. Reference may be made to a recent review by Peters (1937a) on this subject. The subject is also reviewed briefly by Silberschmidt (1936), where full references to the literature on the action of vitamin B_1 in general metabolism are given.

There seems to be no doubt that the brain tissue of pigeons suffering from avitaminosis B_1 respires at a smaller rate in presence of glucose or lactate than normally (Gavrilescu *et al.*, 1932) and that the addition of vitamin B_1 to the brain tissue *in vitro* restores the ability of the brain to respire at a normal rate. When such a brain respires in a lactate medium, pyruvate appears at considerably higher concentrations than with a normal brain (Peters and Thompson, 1934), and the addition of vitamin B_1 to the system causes this pyruvate to disappear. Moreover the minced brain tissue of an avitaminous B_1 pigeon respires at a lesser rate in a pyruvate medium than in such a medium containing added vitamin B_1. Pyruvic acid was the first substance proved to disappear as a result of the action of vitamin B_1. The phenomenon does not only apply to pigeon brain; it applies also to rat brain (O'Brien and Peters, 1935) and to chicken brain (Sherman and Elvejhem, 1936a).

Galvao and Pereira (1936) show that with rats suffering from avitaminosis B_1 the presence of lactate has less effect on the oxygen uptake by the cerebrum than is normally the case. The respiration of the brain stem is unaffected.

Galvao and Florence (1934) have found that the reduction of methyl-

ene blue by lactate is decreased from the normal rate when brain tissue of chickens suffering from avitaminosis B_1 is used.

The results of the investigations of the Oxford school of workers have led to the conclusion that vitamin B_1 is in some way specifically related to the oxidation of pyruvic acid (Peters, 1936; see also Sherman and Elvejhem, 1936b). This result has been amply confirmed by other workers (e.g., Lipmann, 1937; Westenbrink and Polak, 1937). Sinclair (1933) found that the respiratory quotient of brain tissue from polyneuritic pigeons is low and is raised nearly to the normal value by the addition *in vitro* of vitamin B_1.

The amount of vitamin B_1 in brain tissue has been calculated to be 1 unit per gram (Passmore, Peters and Sinclair, 1933).

The mode of action of vitamin B_1 is unknown. Its specificity of action was early shown by Gavrilescu *et al.* (1932) in the fact that succinate oxidation was normally oxidized by the brain tissue of a polyneuritic pigeon (confirmed by Galvao and Florence, 1934, for chicken brain). Krebs (1936) has stated that the vitamin is concerned with anaerobic breakdown of pyruvic acid but Peters (1936) could not confirm this statement.

The discovery by Lohmann and Schuster (1937) that cocarboxylase is a pyrophosphoric ester of vitamin B_1 led to the suggestion that the accumulation of pyruvic acid during the respiration of a polyneuritic pigeon brain is due to lack of this coenzyme. Peters (1937b) finds that although vitamin B_1 is rapidly phosphorylated by brain tissue, cocarboxylase has less effect than vitamin B_1 in increasing the oxygen uptake of polyneuritic pigeon brain. It is therefore unlikely, but it is not impossible, that vitamin B_1 exerts its effect on pyruvate oxidation as a phosphoric acid ester.

Oxidation of succinic acid in brain. This oxidation which, as has already been stated, is unaffected by the absence of vitamin B_1 from brain has been studied by a number of workers (Ashford and Holmes, 1930; Quastel and Wheatley, 1932; Peters and Sinclair, 1933). Succinic acid is rapidly oxidized by brain both aerobically and anaerobically (by methylene blue or by ferricyanide). It was shown by Quastel and Wheatley (1931) that the oxidation of succinate by brain proceeds to fumarate and *l*-malate, the further removal of the latter substance being relatively very slow. This has been confirmed by Elliott *et al.* (1937). The oxidation of succinate is independent or partially independent of the oxidations of glucose or lactate. The presence of malonate greatly inhibits the oxidation of succinate by brain. According to Greville

(1936) malonate brings about a decrease of the oxygen consumption of rat brain in presence of glucose, pointing possibly to succinic acid being involved in the respiration of glucose; the further addition of fumarate does not, however, completely neutralize this inhibition as should occur if the "succinic acid cycle" of Szent-Gyorgyi and his colleagues applies to brain respiration.

OXIDATION OF OTHER SUBSTRATES BY BRAIN. *l-Malate.* The addition of this substance to brain slices has a small effect in increasing the Q_{O_2} (see Elliott *et al.*, 1937). It is definitely oxidized in presence of brain slices anaerobically, by ferricyanide so long as cozymase is added to the system (Quastel and Wheatley, 1938).

α-Glycerophosphate. This substance reduces methylene blue in presence of brain tissue (Ashford and Holmes, 1931; Davies and Quastel, 1932).

Peters and Sinclair (1933) found that α-glycerophosphate is oxidized by minced pigeon brain, the mechanism being independent of lactate oxidation. β-Glycerophosphate is not oxidized by the tissue. Johnson (1936) has found that the aerobic removal of α-glycerophosphate in presence of minced pigeon brain is accelerated by the addition of sodium pyrophosphate. It is not influenced by the presence of pyruvate. Reducing substances are formed when α-glycerophosphate is oxidized by brain and from solutions in which this oxidation has proceeded the 2:4 dinitrophenylbis-hydrazone of methylglyoxal and the 2:4 dinitrophenyl hydrazone of a compound of probably six carbon atoms have been prepared. A study of α-glycerophosphate dehydrogenase has been made by Green (1936) who finds that in the rabbit the highest concentration of the enzyme is in the brain. The presence of α-glycerophosphate (0.03 *M.*) increases Q_{O_2} of rat brain cortex from 3.0 to 8.4, the effect being considerably less than that of glucose (Quastel, Tennenbaum and Wheatley, 1936).

Acetate. The addition of this substance has little or no effect on the respiration of brain (Elliott, Greig and Benoy, 1937; Weil-Malherbe, 1937a; Mann, Tennenbaum and Quastel, 1938).

Acetoacetate. Acetoacetate produces a small increase of the respiration of brain (McGowan and Peters, 1937; Krebs and Johnson, 1937; Mann, Tennenbaum and Quastel, 1938). It undergoes some reduction to β-hydroxybutyrate under anaerobic conditions (Weil-Malherbe, 1938).

β-Hydroxybutyrate. This substance is oxidized by rat brain cortex acetoacetate being formed (Jowett and Quastel, 1935).

α-Ketoglutarate. α-Ketoglutaric acid is oxidized by brain slices (Krebs and Johnson, 1937) and by minced pigeon brain (McGowan and Peters, 1937) but there is little or no accelerating effect of vitamin B_1 on its oxidation (in contrast to that with pyruvic acid) when polyneuritic pigeon brain is used. α-Ketoglutaric acid, like pyruvic acid, gives rise to succinic acid when incubated anaerobically with minced pigeon brain (Weil-Malherbe 1937b). It is reduced in brain to 1-α hydroxyglutarate.

α-Ketoadipate. α-Ketoadipic acid causes no change in the respiration of minced pigeon brain (McGowan and Peters, 1937).

Acetopyruvate. This substance is utilized both aerobically and anaerobically by pigeon brain (Krebs and Johnson, 1937c).

α-Hydroxyacetoacetate. This substance is not affected by rat brain slices (Weil-Malherbe, 1938).

Phosphoglycerate. Phosphoglyceric acid leads to a slight increase in the Q_{O_2} of guinea-pig brain cortex slices (Jowett and Quastel, 1937a). This fact is of interest since if phosphoglycerate behaves with brain as it does with muscle, it should be transformed to pyruvate and hence raise the respiration of brain tissue appreciably. This does not occur; nor does it raise the respiration in presence of α-glycerophosphate.

Gluconate. Sodium gluconate does not increase the respiration of minced guinea-pig brain tissue (Quastel and Wheatley, 1932).

Citrate. Sodium citrate was shown by Thunberg (1923) to be a hydrogen donator in presence of nerve tissue. It actively reduces methylene blue in presence of minced ox brain tissue (Davies and Quastel, 1932). Its Michaelis constant is shown in table 10. Its dehydrogenase is apparently very labile compared with that for succinate, lactate or α-glycerophosphate.

Hexosediphosphate, hexosemonophosphate (Robison ester), glycerol. These substances are oxidized by brain cortex slices but at relatively slow rates compared with that of glucose.

Alcohol. Robertson and Stewart (1932) have shown that ethyl alcohol in small concentrations may undergo oxidation in the presence of brain tissue; in large concentrations it acts as a narcotic presumably by displacing the metabolites present in the brain from the active surfaces.

Himwich and his colleagues (1933) have made observations of the R. Q. of the cerebral cortex of the rat after treatment with alcohol. The R. Q. was found to drop from a value of unity to an average value of 0.87.

Inositol. This substance does not increase the respiration of rat or rabbit brain (Young, 1936).

Fatty acids. Guinea-pig brain cortex slices are unable to oxidize lower fatty acids (Quastel and Wheatley, 1933).

Amino acids. Glutamic acid (0.05 M) is oxidized at a comparatively low rate by either fresh minced brain tissue or by tissue which has been allowed to respire for three hours before the amino acid is added (Quastel and Wheatley, 1932). Krebs (1935) has confirmed the fact that glutamic acid is oxidized by brain and has also shown that brain slices synthesize glutamine from glutamic acid and ammonia so long as energy is supplied by glucose oxidation in brain. Weil-Malherbe (1936) has found that whilst 1 (+) glutamic acid is burned by the brain, d (−) glutamic acid is not oxidized. Apparently 1 (+) glutamic acid is the only amino acid oxidized by brain. It is oxidized first to α-ketoglutaric acid, whose further breakdown can be inhibited by the addition of arsenite to the brain tissue. The Q_{O_2} of rat brain cortex is increased from 2.9 to 8.0 by the presence of 0.02 M 1 (+) glutamate (Jowett and Quastel, 1937b).

Amines. An amine oxidase exists in brain which brings about the oxidation of the aliphatic amines, butylamine, amylamine, isoamylamine and heptylamine. Propylamine, ethylamine and methylamine are not oxidized. This amine oxidase will also oxidize tyramine and indolethylamine (Pugh and Quastel, 1937) and adrenaline and various other aromatic amines (Blaschko *et al.*, 1937). The amines in question are oxidized by brain tissue to the corresponding aldehyde, ammonia being liberated. The activity of amine oxidase is inhibited by amines not oxidized by brain, e.g., ephedrine (Blaschko *et al.*, 1937), and by benzedrine (Pugh and Quastel, unpublished). The question has arisen as to whether some of the clinical effects of ephedrine administration are due to its inhibition of amine oxidase (see Gaddum, 1938).

It should be emphasized that no substrates have yet been found which equal either glucose, lactate or pyruvate in increasing and maintaining at a constant rate, the respiration of brain tissue *in vitro.*

Influence of the ionic environment on brain respiration. Ashford and K. C. Dixon (1935) have observed that the presence of 0.1 M KCl greatly increases lactic acid production in the presence of oxygen but decreases it in the presence of nitrogen. The total oxygen uptake is also increased by the presence of 0.1 M KCl. According to K. C. Dixon (1936) the addition of potassium chloride raises the aerobic destruction of glucose by brain to the anaerobic level. A careful investigation of

the phenomenon by Dickens and Greville (1935), working with rat brain cortex slices, shows that the presence of 0.1 M KCl increases the respiration of brain tissue whether glucose, lactate, pyruvate or fructose is the substrate. Moreover all the alkali metal chlorides increase the respiration of brain tissue when added in 0.1 M concentration to the medium. The order of activity is Li < Na < Rb < Cs < K. Results of a similar description have been obtained by K. C. Dixon and Holmes (1935). Dickens and Greville state that when brain gray matter is respiring in a salt medium the respiration is steadiest when Na, K and Ca ions are present. K^+ tends to increase the respiration, the bivalent cations Ca and Mg to decrease it. These changes in ionic concentration, which have large effects on brain metabolism, have no such effects on other normal tissues investigated—these being kidney cortex, testis, liver, yolk sac and retina.

TABLE 14

ANIMAL	Q_{O_2}	Q_{O_2} IN PRESENCE OF NARCOTIC
Guinea pig	3.9	0.37
Cat (grey matter)	2.8	0.27
Rabbit	2.8	0.47
Mouse	3.2	0.94
Calf (grey matter)	3.9	0.97
Sheep (grey matter)	3.2	0.80

EFFECTS OF DRUGS AND POISONS ON RESPIRATION IN THE CENTRAL NERVOUS SYSTEM. *Narcotics*. The large literature bearing on narcotic action cannot be dealt with here. It has been summarized by Winterstein (1926, 1936) and Henderson (1930), (see also Pick, 1937).

Bülow and Holmes (1932) have stated that the oxygen uptake of minced brain tissue is unaffected by the presence of anesthetic gases such as nitrous oxide and acetylene. It may be shown clearly, however, that the oxygen uptake of brain tissue is very considerably diminished by the presence of many different types of narcotics.

Table 14 shows the effects of a 0.4 per cent solution of somnifaine (a drug composed of the diethylamine salts of veronal and allylisopropyl barbituric acid) on the respiration of fresh minced brain tissue in a phosphate-saline (glucose free) medium (Quastel and Wheatley, 1932b).

Whereas the narcotics had highly inhibitive effects on the respiration of brain they had none at the concentrations used on an actively respiring yeast (see table 15).

The results of narcotics cited above have been found with minced brain tissue. They apply equally to brain tissue in the form of cortex slices. This is seen in table 16 showing the inhibitive action of chloretone (0.002 M) on the respiration of guinea-pig brain cortex slices.

The inhibitive effect of the narcotic is not due to irreversible damage of the brain tissue as shown by the fact that on washing the brain slices which have been exposed to the narcotic in a glucose phosphate medium the initial respiration of the brain is largely recovered. This reversi-

TABLE 15

NARCOTIC (0.3 PER CENT)	μL O_2 UPTAKE BY YEAST (0.05 GRAM) IN 2 HOURS	μL O_2 UPTAKE BY GUINEA PIG BRAIN (MINCED) 0.5 GRAM IN 2 HOURS
None	252	840
Ethylurethane	251	670
Allylisopropyl-barbituric acid	290	492

TABLE 16

Rate of O_2 uptake by guinea-pig brain cortex slices in a phosphate-saline-glucose medium in the absence and in the presence of chloretone (0.002 M)

	Q_{O_2}				AVERAGE Q_{O_2}	PER CENT DECREASE IN Q_{O_2} DUE TO NARCOTIC
	min.	*min.*	*min.*	*min.*		
Time from commencement of experiment	15	30	45	60		
In the absence of the narcotic	14.7	15.6	15.9	15.9	15.5	
In the presence of the narcotic	4.8	5.1	4.8	5.3	5.8	68

bility in action is shown in table 17 (Quastel and Wheatley, 1934). The following facts are noteworthy:

1. The Q_{O_2} of brain slices remains nearly constant throughout the experimental period.

2. The effect of the narcotic is rapid, the Q_{O_2} in presence of the narcotic reaching its equilibrium value within 15 minutes.

3. The Q_{O_2} does not fall progressively with time as the brain slices continue to be exposed to the narcotic. Were the narcotic a "cell poison" having irreversible action it would be expected that a progressive fall in respiration would occur.

4. The effect of washing brain slices previously immersed in the narcotic solution and of adding the washed slices to a fresh glucose

medium is to raise the Q_{O_2} to a level not far removed from that obtained with brain slices to which no narcotic had been added. With such washed brain slices a constant Q_{O_2} is rapidly reached showing the attainment of a new equilibrium.

Two-hundredths per cent (0.001 M) chloretone exercises an inhibition of the respiration of guinea-pig brain cortex slices of 50 per cent the inhibition being largely removed (to the extent of 70 per cent) by washing the slices. Eight-hundredths per cent luminal (0.0035 M) inhibits the respiration of cortex slices by 40 per cent the inhibition being decreased 80 per cent by washing the slices even after they had been exposed to luminal for 2 hours.

Wortis (1935) has used two methods to measure the effects of sedatives on oxidation by brain tissue:

1. Injection of different drugs into normal white rats was carried out until narcosis resulted. The animal's brain was then removed and the

TABLE 17

	Q_{O_2} IN PHOSPHATE GLUCOSE MEDIUM (AFTER WASHING THE CORTEX SLICES PREVIOUSLY EXPOSED TO CHLORETONE 0.002 M)							AVERAGE PER CENT DECREASE IN Q_{O_2}
	min.	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	
Time from the commencement of the experiment	15	30	45	60	75	90	120	
No narcotic previously added (control)	14.4	15.5	14.9	13.8	13.8	14.2	14.2	
0.002M chloretone previously added (producing 68% drop in Q_{O_2})	10.0	12.3	13.3	11.9	12.2	11.9	12.0	16

respiration of the brain tissue measured manometrically. Morphine, butyl-bromallyl-barbituric acid and sodium amytal did not appear to affect R. Q. or O_2 consumption when the results were compared with normal brain tissue. This negative result is to be expected since, as has been pointed out, the effects of the narcotics are reversible. Immersion of brain tissue containing a narcotic (at a sufficiently low concentration) into a narcotic free medium results in a washing out of the narcotic and the consequent resumption of normal respiration.

2. Normal rat brain tissues (minced) was immersed in fluids containing various drugs at different concentrations and the R. Q. and the O_2 consumption were subsequently measured over a period of 2 hours. Cocaine, luminal, caffeine, sodiobenzoate and sodium bromide reduced the oxygen consumption of brain tissue.

Sherif (1930) has shown that cocaine, novocaine and urethane decrease the rate of oxygen consumption of nerve tissue, the first named being the most and the last the least active.

Quastel and Wheatley (1932b) as a result of investigation of the respiration of guinea-pig brain tissue in presence of various metabolites and narcotics came to the following conclusions:

1. Narcotics in general inhibit the oxidation by brain of glucose, sodium lactate and sodium pyruvate. They have some inhibitory power on the oxidation of glutamic acid. They do not inhibit the oxidation by brain of sodium succinate or of p-phenylenediamine.

2. Among narcotics of the same chemical type, those with the greater hypnotic activity have the greater inhibitive power.

It was suggested that a view of the mechanism of narcosis fitting most closely to the facts is that a narcotic is adsorbed from the blood at a

TABLE 18

SUBSTRATE	Q_{O_2} WITHOUT NARCOTIC	PER CENT INHIBITION OF Q_{O_2} FOUND WHEN 0.08 PER CENT LUMINAL IS PRESENT
Nil	2.89	5
Glucose 0.01M	12.20	55
d-Lactate 0.02M	13.55	35
Pyruvate 0.02M	11.10	27
Glutamate 0.02M	8.03	15
Succinate 0.02M	9.53	Nil

specific area or centre of the nervous system. Here it brings about a diminution of the ability of the cells constituting the nervous centre to oxidize pyruvic acid, lactic acid or glucose. The access—or activation of oxygen—is quite unimpaired as shown by the lack of effect of the narcotic on the oxidation of sodium succinate or p-phenylenediamine. Narcosis—or a depression of the normal functional activity of the nervous center in question may then ensue. The specific effects of narcotics will depend on the specificities of adsorption in various parts of the nervous system.

Jowett and Quastel (1937b) studying rat and guinea-pig brain cortex slices find that the respiration is inhibited by evipan, luminal, chloretone and ether in presence of glucose, sodium lactate and sodium pyruvate. Table 18 illustrates results obtained with rat brain cortex slices and 0.08 per cent luminal.

The sensitivity of brain respiration, as compared with that of other organs, to a narcotic is shown in table 19.

Jowett and Quastel found that the inhibition of respiration by narcotics tends to be independent of time when the potassium ion concentration of the medium is at the normal serum level, but that it increases rapidly with time when the potassium ion concentration is low (0.002 M). They also found that narcotics bring about inhibition of respiration by slices of liver, kidney and diaphragm, both when no substrates are added and in the presence of some substrate such as pyruvate. The effects of ether on brain cortex slice respiration are shown in table 20 (Jowett and Quastel, 1937c).

The inhibitory action of ether on the oxidation of glucose or of lactate has a large temperature coefficient (Q_{10° = 6, between 37° and 42°).

TABLE 19

Effect of 0.033 per cent evipan on respiration of guinea pig tissues in presence of glucose

TISSUE (SLICES)	Q_{O_2} ALONE	Q_{O_2} IN PRESENCE OF EVIPAN	EFFECT OF NARCOTIC
			per cent
Kidney cortex	15.2	15.95	+5
Liver	4.25	4.15	−2
Testis	8.65	7.25	−16
Spleen	7.7	6.4	−17
Brain cortex	14.2	9.5	−33

It is clear that ether, when compared with other narcotics such as the barbiturates, has much more pronounced irreversible effects. The inhibitory effect of ether on glucose oxidation by cerebral cortex at the anesthetic concentration is of the order of 10 per cent (Jowett and Quastel, 1937c).

Table 21 (Jowett, 1938) summarizes the results of Jowett and Quastel (1937b, c) and of Jowett (1938) on the effects of narcotics on brain cortex respiration, in the presence of glucose, at the concentrations required to induce narcosis.

The data in table 21 are sufficient to show that a definite inhibition of respiration is produced by concentrations of the order producing deep narcosis.

The facts given above, which show the specificity of inhibition, effected by narcotics on the respiratory processes of the central nervous

system, acquire a special significance when it is considered, as has already been pointed out, that carbohydrate breakdown occupies a dominant position in the metabolism of the central nervous system.

TABLE 20

ANIMAL	SUBSTRATE 0.02 M	PERCENT INHIBITION AFTER 80 MINUTES IN PRESENCE OF	
		(a) 0.05–0.055 M ether	(b) 0.10–0.11 M ether
Rat	Glucose	52	79
	Lactate	32	
	Fructose	32	
	Glutamate	18	50
	Succinate	0	
Guinea pig	Glucose	60	80
	Lactate	29	
	Pyruvate	29	
	Galactose		42
	α Glycerophosphate	0	
	Hexosediphosphate	0	

TABLE 21

NARCOTIC	ANIMAL	NARCOTISING CONCENTRATION (M)	PER CENT INHIBITION OF RESPIRATION DUE TO THE NARCOTISING CONCENTRATION OF NARCOTIC	RATIO: CONCENTRATION REQUIRED TO PRODUCE 15 PER CENT INHIBITION, DIVIDED BY NARCOTISING CONCENTRATION
Ethyl urethane	Rat	0.022	6	2.9
Magnesium ion	Rabbit	0.005*	12	1.2
Magnesium ion	Rat	0.005	13	1.2
Chloral hydrate	Rat	0.0013	10	1.6
Luminal	Rat	0.00079	15	1.0
Chloretone	Rat	0.0010	20	0.7
Evipan	Guinea pig	0.00062	17	0.9
Avertin	Rat	0.00106	31	0.4
Chloretone	Guinea pig	0.0010	32	0.4

* Excess above normal.

It appears probable from these results that narcotics bring about a local anoxemia in those parts of the nervous system where they are adsorbed, owing to their interfering with the oxidation of glucose at the nervous center in question. The access of oxygen however is quite un-

impaired. It has to be borne in mind that the inhibition of respiration to be expected during narcosis as a mean effect spread over the whole of the gray matter of the cerebral cortex is not necessarily large. The effects of narcotics on function are often localized, and the effects on oxidation in the brain may also be localized.

Gross and Pierce (1935) have examined the effects of morphine on the oxidation of glucose by the brain tissue of normal rats and of rats made tolerant to morphine by daily administration of the drug over a period of three weeks. They found that the degree of inhibition by added morphine of the respiration of brain tissue of tolerant animals in the presence of glucose was less than that which obtained in the case of non tolerant animals.

Hundhausen (1938) could find no difference between the inhibitive effects of chloralhydrate, or luminal on the respirations of brain cortex or brain stem.

Alexander and Cserna (1913) and Yamakita (1922) found very large decreases in oxygen utilization by the brain *in vivo* when narcosis was induced by ether (see also Lázló, Urban and Weissenberg, 1935). It is clear, however, from what has been said that large decreases in oxygen utilization by the brain as a whole are not necessarily to be expected when narcosis is induced. Local decreases, at particular parts of the nervous system, need not affect markedly the oxygen consumption of the brain as a whole.

Narcotics greatly inhibit anaerobic oxidation of glucose, fructose, mannose and lactate (Davies and Quastel, 1932) in presence of brain tissue. A competition takes place between the narcotic and substrate (lactate) for the enzyme system involved. Anaerobically, however, narcotics appear to have less inhibitive effects on oxidations by the brain than under aerobic conditions.

The pathological effects in the central nervous system following the administration of hypnotic drugs have been described by Mott, Woodhouse and Pickworth (1926).

Amines. Quastel and Wheatley (1933b) have found that amines, chiefly derived from tyrosine, tryptophane and leucine, inhibit oxidative processes in the brain *in vitro*. There are several similarities between the action of (certain) amines and that of narcotics on brain oxidation. While cadaverine, putrescine, neurine and ethylamine have little action (at the concentrations used) on the oxidations of glucose and sodium lactate, tyramine, β-phenylethylamine, and β-phenyl-β-hydroxyethylamine have relatively large effects. Mescaline (β:3:4·5-trimethoxy-

phenylethylamine), well known for its production of visual hallucinations, has similar large effects. Indole, skatole, isoamylamine have highly inhibitory actions but histamine is without effect. With the exception of tyramine none of the amines tested affects appreciably the oxidation of sodium succinate.

Studies with guinea-pig brain cortex slices show the inhibitory action of mescaline on respiration to be reversible (Quastel and Wheatley, 1934). No reversibility however was found with indole. This may mean that indole has an irreversible damaging effect on the brain cells but it seems as likely that the failure to find reversibility was due to inadequate washing of the brain cortex slices.

Pugh and Quastel (1937) have examined the effects of a number of amines on the respiration of rat brain cortex slices in a glucose medium

TABLE 22

Respiration of brain cortex slices in a glucose medium in presence of tyramine and indolethylamine

AMINE	ANIMAL	Q_{O_2} (OVER 1ST HR)		Q_{O_2} (OVER 2ND HR)	
		Amine absent	Amine present	Amine absent	Amine present
Tyramine 0.0052 M.	Rat	15.5	9.1	12.2	4.6
	Guinea pig	19.6	12.1	16.4	4.7
	Sheep	7.6	4.5	5.0	3.1
Indolethylamine 0.0066 M.	Rat	14.6	11.7	12.3	3.9
	Guinea pig	19.6	11.1	16.4	4.0
	Sheep	7.6	4.4	5.0	2.1

and, in addition to confirming the results already quoted, find that indolethylamine and benzedrine exert pronounced inhibitory actions. Table 22 summarizes some of their results.

It is of interest that many of these amines, which have such inhibitory effects on brain respiration in presence of glucose, should be oxidized by an enzyme (amine oxidase) present in brain as well as in other organs.

It is also of importance to record the fact that a number of amines—phenylethylamine derivatives—give rise to catatonia when injected into animals (De Jong and Baruk, 1930; De Jong, 1932; Noteboom, 1934). Whether such catatonic symptoms are related to a depression of respiration in some part of the nervous system is a problem still to be investigated.

Iodoacetate. This substance while inhibiting greatly the oxidation of glucose by brain has little effect on that of lactate. Krebs (1931) gives the following figures for rat brain cortex:

	GLUCOSE, 0.2%	*dl*-LACTATE, 0.2%	GLUCOSE, 0.2%	*dl*-LACTATE, 0.2%
	M CONCENTRATION OF IODOACETATE			
	0	0	5×10^{-4}	5×10^{-4}
Q_{O_2} (over first 30 minutes)..	14.3	13.9	2.0	14.0

According to Quastel and Wheatley (1932) iodoacetate (2.5×10^{-4} M) inhibits the oxidation of glucose, fructose and mannose by guinea-pig brain but has little effect on that of *dl*-lactate, pyruvate, glutamate or succinate. Peters, Rydin and Thompson (1935) find that, with pigeon brain, iodoacetate diminishes the accelerating action of vitamin B_1 on pyruvic acid oxidation by avitaminous tissue. Peters (1936b) finds that dichlor-diethylsulphone also has an effect similar to that of iodoacetate.

dl-Glyceraldehyde. This substance (0.01 M) greatly inhibits the respiration of brain in presence of glucose, though it has little effect in absence of glucose (Mann *et al.*, 1938; Baker, 1938).

Fluoride. Fluoride inhibits glucose oxidation to a greater extent than that of lactate (Holmes, 1930) and according to Peters, Rydin and Thompson (1935) it inhibits the accelerating action of vitamin B_1 on pyruvic acid oxidation.

Cardiazole (metrazol, pentamethylenetetrazol). Cardiazole, which has has now come into prominence, as a convulsive reagent in the treatment of schizophrenia has according to Wortis (1938) no effect on the respiration of brain tissue *in vitro* when respiring in a glucose medium. Himwich, Bowman, Wortis and Fazekas (1938) have shown that during cardiazole convulsions, an anoxemia develops. During various stages of the convulsions the oxygen saturation of the blood falls to 42 per cent. Even in the first moments after the convulsions cease the oxygen tension of the arterial blood does not attain normal value. It appears, therefore, that the effect of the convulsions is to reduce the oxygen supply to the nervous system (see also Himwich *et al.*, 1937).

It is worth pointing out, at this juncture, that in the present most widely used active treatments of mental disorder, namely, prolonged narcosis treatment, insulin "shock" therapy, and cardiazole convulsive treatment, there appears to be a common factor—an interference with

the respiratory metabolism of the central nervous system. In narcosis treatment there is probably a diminished ability to burn glucose at those nervous centers affected by the narcotic; in insulin "shock" therapy there is a diminished quantity of glucose available for all the nervous system, and in cardiazole convulsion therapy, the access of oxygen itself to the nervous system is cut down.

p-Aminonaphthol. This substance at a concentration of 10^{-4} M inhibits brain respiration by 40 per cent (Bernheim *et al.*, 1937).

Dinitro-o-cresol. Dinitrophenols increase the respiration of normal tissues (Dodds and Greville, 1933; Ehrenfest and Ronzoni, 1933). Greville (1936) describes an experiment showing that the Q_{O_2} of rat brain cortex in presence of glucose is raised from $Q_{O_2} = 13.1$ to $Q_{O_2} = 39.0$ by the addition 10^{-5} M dinitro-o-cresol. The addition of malonate abolishes the accelerating action of dinitro-o-cresol.

Pyocyanine. Young (1937) has shown that the presence of pyocyanine (10^{-3} M $-$ 10^{-5} M) increases markedly the respiration of rat brain cortex in presence of glucose. Oxidation in presence of lactate, fructose and pyruvate are also increased, but when no substrate is added no increased respiration is noted.

Dyestuffs. The accelerative actions of dyes such as thionine, brilliant cresyl blue and the inhibitive action of other dyestuffs on brain cortex respiration in presence of glucose have been described by Dickens (1936). Elliott and Baker (1935) have also contributed to this subject, making use of 2-6-dichlorophenolindophenol and dinitrocresol.

Fractions from B. aertrycke. Toxic fractions from *Bact. aertrycke* have the power of greatly increasing the oxygen uptake of rabbit brain suspensions in the presence of glucose, but they have no effect in the absence of added substrate (Delafield and Smith, 1936). These toxic fractions slightly diminish the O_2 uptake of brain suspensions in presence of succinate.

Lead. According to Dolowitz, Fazekas and Himwich (1937) 5 mgm. lead acetate per 100 grams tissue reduce the oxygen consumption of brain tissue *in vitro.*

Acetylcholine synthesis in the central nervous system. It has been shown recently (Quastel *et al.*, 1937; Mann *et al*, 1938) that acetylcholine synthesis in the brain depends upon the existence of an intact respiratory metabolism. Under anaerobic conditions the synthesis of this substance does not take place, and under aerobic conditions either glucose, lactate or pyruvate is essential for the formation of acetylcholine to occur.

Content of vitamins concerned with respiration and of glutathione in the

central nervous system. *Vitamin B_1.* The content of this substance in the brain has already been mentioned.

Lactoflavin (*vitamin B_2*). This vitamin is present in brain and its amount is given as 1γ–5γ per gram in beef brain (Euler and Adler, 1934), as 2.2γ per gram in gray matter of sheep brain and as 1γ per gram in the white matter (see Gourévitch, 1937). Charite and Khaustov (1935) state that human brain contains 1.5γ per gram. Neurological manifestations in vitamin B_2 deficiency are described by Zimmermann *et al.*, 1937.

Ascorbic acid. Bessey and King (1933) give the following values of the ascorbic acid content of brain. Rat 0.36 mgm/gram; rabbit 0.31 mgm/gram; chicken 0.33 mgm/gram; hog 0.18 mgm/gram. Plaut and Bülow (1934, 1935) find brain rich in ascorbic acid and quote the figures given in table 23, which show the variation of ascorbic acid content of various parts of the brain with age.

TABLE 23

	NUMBER OF CASES	AGE	ASCORBIC ACID IN		
			Cortex	Cerebellar cortex	Spinal cord
			mgm. per cent	*mgm. per cent*	*mgm. per cent*
Fetus	7	3–5 months	65	37	16
Infant	6	1½–9 months	23	31	17
Children	4	2¼–8½ years	18	23	16
Adult	16	25–56 years	10	13	13
Aged	12	65–82 years	7.5	9	11

Wortis, Wortis and Marsh (1938) have recently confirmed the finding of Plaut and Bülow (1936) that ascorbic acid in the cerebrospinal fluid is an accurate index of the amount present in the blood. They find that the R. Q. of brain of scorbutic guinea-pig brain is unity and that such brain respires at the same rate as the normal. Gluck and Biskind (1935), studying the pituitary, find that the concentrations of ascorbic acid in the pars nervosa, intermedia and distalis are in the ratio 1:4.7:3.

The details and mechanisms of action of lactoflavin or of ascorbic acid in the respiratory processes of the nervous system are at present unknown.

Glutathione. Glutathione occurs in rabbit brain to the extent of 75 mgm. per cent wet weight in gray matter and 52 mgm. per cent in white matter (Holmes, 1926). Possibly part of this is actually ascorbic

acid since iodine titrations were used. Boyland (1933) gives the glutathione content of rat brain as of the order 50 mgm. per cent whilst according to Wachholder, Anders and Uhlenbrook (1935) the glutathione content of rabbit brain is 63–95 mgm. per cent.

REFERENCES

Alexander, F. G. and S. Cserna. Biochem. Ztschr. 53: 100, 1913.

Alt, H. L. Biochem. Ztschr. 221: 498, 1930.

Ashford, C. H. and K. C. Dixon. Biochem. J. 29: 157, 1935.

Ashford, C. H. and E. G. Holmes. Biochem. J. 23: 748, 1929.
Biochem. J. 25: 2028, 1931.

Baker, Z. *Biochem J. 32: 332, 1938.*

Baker, Z., J. F. Fazekas and H. E. Himwich. J. Biol. Chem. 125: 545, 1938.

Banga, I., L. Schneider and H. Szent-Gyorgyi. Biochem. Ztschr. 240: 454, 1931.

Barcroft, J. The respiratory function of the blood. II. Haemoglobin. Cambridge, 1928.
The architecture of physiological function. Cambridge, 1934.
Lessons from high altitudes. Cambridge, 1936.

Behnke, A. R., R. M. Thomson and E. P. Motley. Am. J. Physiol. 110: 565; 112: 554, 1935.

Benoy, M. P. and K. A. C. Elliott. Biochem. J. 31: 1268, 1937.

Bernheim, F., M. L. C. Bernheim and H. O. Michel. J. Pharmacol. and Exper. Therap. 61: 311, 1937.

Bert, P. La pression barometrique. Paris, 1878.

Bessey, O. A. and C. G. King. J. Biol. Chem. 103: 687, 1933.

Blaschko, H., D. Richter and H. Schlossmann. Biochem. J. 31: 2187, 1937.

Boyland, E. Biochem. J. 27: 802, 1933.

Braunstein, A. E. and M. G. Kritzmann. Enzymologia 2: 129, 1937.

Brookens, N. L., L. Ectors and R. W. Gerard. Am. J. Physiol. 116: 16, 1936.

Bülow, M. and E. G. Holmes. Biochem. Ztschr. 245: 459, 1932.

Chang, T. H. and R. W. Gerard. Am. J. Physiol. 97: 511, 1931.

Charite, A. J. and N. W. Khaustov. Biochem. J. 29: 34, 1935.

Cobb, S. and H. S. Forbes. New England J. Med. 216: 99, 1937.

Cobb, S. and F. Fremont-Smith. Arch. Neur. Psychiat. 26: 731, 1931.

Cobb, S. and J. H. Talbot. Trans. Assn. Am. Physicians 42: 255, 1927.

Cohen, M. B. and R. W. Gerard. Am. J. Physiol. 119: 34, 1937.

Dameshek, W., A. Myerson and C. Stephenson. Arch. Neur. Psych. 33: 1, 1935.

Davies, D. R. and J. H. Quastel. Biochem. J. 26: 1672, 1932.

Delafield, M. E. and H. A. Smith. Brit. J. Exper. Path. 17: 379, 1936.

Dewan, J. G. and D. E. Green. Biochem. J. 32: 626, 1938.

Dickens, F. Biochem. J. 30: 661, 1936a.
Biochem. J. 30: 1064, 1936b.

Dickens, F. and G. D. Greville. Biochem. J. 27: 832, 1933.
Biochem. J. 29: 1468, 1935.

Dickens, F. and F. Šimer. Biochem. J. 24: 905, 1930a.
Biochem. J. 24: 1301, 1930b.
Biochem. J. 25: 985, 1931.

DIXON, K. C. Biochem. J. 30: 1479, 1936.
DIXON, K. C. AND E. G. HOLMES. Nature 135: 995, 1935.
DIXON, M. Manometric methods. Cambridge, 1934.
DIXON, M. AND K. C. ELLIOTT. Biochem. J. 23: 815, 1929.
DIXON, T. F. AND A. MEYER. Biochem. J. 30: 1577, 1936.
DODDS E. C. AND G. D. GREVILLE. Nature 132: 966, 1933.
DOLOWITZ, D. J., J. F. FAZEKAS AND H. E. HIMWICH. J. Ind. Hyg. 19: 93, 1937.
EHRENFEST AND RONZONI. Proc. Soc. Exper. Biol. Med. 31: 318, 1933.
ELLIOTT, K. A. C. AND Z. BAKER. Biochem. J. 29: 2396, 1935a; Biochem. J. 29: 2433, 1935b.
ELLIOTT, K. A. C. AND G. BANCROFT. Biochem. J. 28: 1911, 1934.
ELLIOTT, K. A. C. AND M. G. GREIG. Biochem. J. 31: 1021, 1937.
ELLIOTT, K. A. C., M. G. GREIG AND M. P. BENOY. Biochem. J. 31: 1003, 1937.
ELLIOTT, K. A. C. AND E. F. SCHRODER. Biochem. J. 28: 1920, 1934.
D'ELSEAUX, F. C. AND H. C. SOLOMON. Arch. Neur. Psych. 29: 213, 1933.
EULER, H. v. AND E. ADLER. Ztschr. physiol. chem. 223: 105, 1934.
EULER, H. v. AND H. HELLSTRÖM. Ztschr. physiol. chem. 252: 31, 1938.
GADDIE, R. AND C. P. STEWART. Biochem. J. 29: 2101, 1935.
GADDUM, J. H. Brit. Med. J. i: 713, 1938.
GALVAO, P. E. AND C. H. FLORENCE. Pflüger's Arch. 233: 714, 1934.
GALVAO, P. E. AND J. PEREIRA. Biochem. Ztschr. 289: 136, 1936.
GAVRILESCU, N., A. P. MEIKLEJOHN, R. PASSMORE AND R. A. PETERS. Proc. Roy. Soc. B. 110: 431, 1932.
GEIGER, A. Biochem. J. 29: 811, 1935.
GELLHORN, E. Am. J. Physiol. 117: 75, 1936.
Ann. Int. Med. 10: 1267, 1937.
GELLHORN, E. AND A. JOSLYN. J. Psychology 3: 161, 1936.
GERARD, R. W. Physiol. Rev. 12: 472, 1932.
Ann. Rev. Biochem. 6: 419, 1937.
GERARD, R. W. AND R. J. SCHACHTER. Proc. Soc. Exper. Biol. Med. 29: 525, 1932.
GIBBS, F. A., E. L. GIBBS AND W. G. LENNOX. Brain 58: 44, 1935a.
Am. J. Physiol. 111: 557, 1935b.
GIBBS, F. A., W. G. LENNOX AND E. L. GIBBS. Arch. Neurol. and Psychiat. 32: 257, 1934.
GILDEA, E. F. AND S. COBB. Arch. Neurol. and Psychiat. 23: 876, 1930.
GOURÉVITCH, A. Bull. Soc. Chim. Biol. 19: 527, 1937.
GREEN, D. E. Biochem. J. 30: 629, 1936.
GREEN, D. E. AND J. BROSTEAUX. Biochem. J. 30: 1498, 1936.
GROSS, E. G. AND I. H. PIERCE. J. Pharmacol. Exper. Therap. 53: 156, 1935.
HAARMAN, W. Biochem. Ztschr. 255: 121, 136, 1932.
HENDERSON, V. E. Physiol. Rev. 10: 171, 1930.
HENDERSON, Y., A. W. OUGHTERSEN, L. A. GREENBERG AND C. F. SEARLE. Am. J. Physiol. 114: 261, 1936.
HEYMANS, C. AND J. J. BOUCKAERT. Compt. Rend. Soc. Biol. 119: 324, 1935.
HIMWICH, H. E., K. M. BOWMAN, J. F. FAZEKAS AND L. L. ORENSTEIN. Proc. Soc. Exper. Biol. Med. 37: 359, 1937.
HIMWICH, H. E., K. M. BOWMAN, J. WORTIS AND J. F. FAZEKAS. J. A. M. A., 1938a. (In the press.)
J. Nerv. Ment. Dis., 1938b. (In the press.)

HIMWICH, H. E. AND J. F. FAZEKAS. Proc. Soc. Exper. Biol. Med. 30: 366, 1932.
Endocrinology 21: 800, 1937.
HIMWICH, H. E., J. F. FAZEKAS AND HURLBURT. Proc. Soc. Exper. Biol. Med. 30: 904, 1933.
HIMWICH, H. E. AND L. H. NAHUM. Am. J. Physiol. 90: 389, 1929.
Am. J. Physiol. 101: 446, 1932.
HIMWICH, H. E., L. H. NAHUM, N. RAKIETEN, J. F. FAZEKAS, D. DU BOIS AND E. F. GILDEA. J. A. M. A. 100: 651, 1933.
HOLMES, B. E. Biochem. J. 20: 812, 1926.
HOLMES, E. G. Biochem. J. 24: 915, 1930.
Biochem. J. 26: 2005, 1932.
Ann. Rev. Biochem. 4: 435, 1935.
HOLMES, E. G. AND C. H. ASHFORD. Biochem. J. 24: 1119, 1930.
HOLMES, E. G. AND B. E. HOLMES. Biochem. J. 21: 412, 1927.
HOU, C. L. J. Orient. Med. 5: 20, 1926.
HUNDHAUSEN, G. Ztschr. ges. Exper. Med. **102**: *477, 1938.*
JOHNSON, R. E. Biochem. J. 30: 33, 1936.
JONG, H. DE. Ztschr. ges. Neurol. Psych. 139: 468, 1932.
JONG, H. DE AND H. BARUK. La catatonie expérimentale par la bulbocapnine. Masson, 1930.
JOWETT, M. J. Physiol. 92: 322, 1938.
JOWETT, M. AND J. H. QUASTEL. Biochem. J. 29: 2182, 1935.
Biochem. J. 31: 275, 1937a.
Biochem. J. 31: 565, 1937b.
Biochem. J. 31: 1101, 1937c.
KERR, S. E. AND M. GHANTUS. J. Biol. Chem. 116: 1, 9, 1936.
KERR, S. E., C. W. HAMPEL AND M. GHANTUS. J. Biol. Chem. 119: 405, 1937.
KERR, S. E. J. Biol. Chem. 123: 443, 1938.
KISCH, B. Biochem. Ztschr. 263: 75, 187, 1933.
KRAINES, S. H. Am. J. Psych. 93: 1435, 1937.
KREBS, H. A. Biochem. Ztschr. 234: 278, 1931.
Biochem. J. 29: 1620, 1951, 1935.
Nature 138: 288, 1936.
Biochem. J. 31: 661, 1937.
KREBS, H. A. AND W. A. JOHNSON. Biochem. J. 31: 645, 1937a.
Enzymologia 4: 148, 1937b.
Biochem. J. 31: 777, 1937c.
KREBS, H. A. AND H. ROSENHAGEN. Ztschr. ges. Neur. Psych. 134: 643, 1931.
KRITZMANN, M. G. Enzymologia 5: 4, 1938.
LAMBERT, E. H. AND E. GELLHORN. Proc. Soc. Exper. Biol. Med. 36: 169, 1937.
LÁZLÓ, D., H. URBAN AND E. WEISSENBERG. Arch. Exper. Path. u. Pharmakol. 179: 266, 1935.
LENNOX, W. G. Arch. Neurol. Psychiat. 26: 719, 1931.
Arch. Neurol. Psychiat. 36: 375, 1936.
LENNOX, W. G. AND BEHNKE. Arch. Neurol. Psychiat. 35: 782, 1936.
LENNOX, W. G. AND E. L. GIBBS. Arch. Neurol. Psychiat. 36: 13, 1936.
LENNOX, W. G., F. A. GIBBS AND E. L. GIBBS. Arch. Neurol. Psychiat. 34: 1001, 1935.
LIPMANN, F. Skand. Arch. f. Physiol. 76: 255, 1937.

LOEBEL, R. O. Biochem. Ztschr. 161: 219, 1925.
LOEVENHART, A. S., W. F. LORENZ, H. G. MARTIN AND J. Y. MALONE. Arch. Int. Med. 21: 109, 1918.
LOEVENHART, A. S., J. Y. MALONE AND H. G. MARTIN. J. Pharmacol. Exper. Therap. 19: 13, 1922.
LOHMANN, K. AND SCHUSTER. Naturwissenschaften 25: 26, 1937.
LONG, C. Biochem. J. 32: 1711, 1938.
LASER, H. Biochem. J. 31: 1677, 1937.
MCFARLAND, R. A. Arch. Psychol., no. 145. Columbia Univ., 1932.
J. Comp. Psych. 23: 191; 24: 147, 1937.
MCFARLAND, R. A., C. H. KNEHR AND C. BERENS. Am. J. Ophthalmology 20: 1204, 1937.
MCGOWAN, G. K. Biochem. J. 31: 1637, 1937.
MCGINTY, D. A. Am. J. Physiol. 88: 312, 1929.
MANN, P. J. G., M. TENNENBAUM AND J. H. QUASTEL. Biochem. J. 32: 253, 1938.
MARSHALL, E. K. AND M. ROSENFELD. J. Pharmacol. Exper. Therap. 57: 437, 1936.
J. Pharmacol. Exper. Therap. 59: 222, 1937.
MEYER, A. Ztschr. ges. Neurol. Psych. 139: 422, 1932.
Ztschr. ges. Neurol. Psych. 143: 333, 1933.
MEYERHOF, O. Die chemische Vorgänge im Muskel. Berlin, 1930.
MEYERHOF, O. AND K. LOHMANN. Biochem. Ztschr. 171: 425, 1926.
MOTT, F. W., D. L. WOODHOUSE AND F. A. PICKWORTH. Brit. J. Exper. Path. 7: 325, 1926.
MYERSON, A. AND R. D. HALLORAN. Am. J. Psychiat. 10: 389, 1930.
Bull. Mass. Dept. Ment. Dis. 15: 9, 1931.
NELSON, M. E. AND C. H. WORKMAN. Iowa State Coll. J. of Sc. 10: 141, 1936.
NOTEBOOM. Proc. Kon. Ak. Wet. Amsterdam. 37: 562, 1934.
O'BRIEN, J. R. AND R. A. PETERS. J. Physiol. 85: 454, 1935.
PAGE, I. H. Chemistry of the brain. C. C. Thomas, 1938.
PASSMORE, R., R. A. PETERS AND H. M. SINCLAIR. Biochem. J. 27: 842, 1933.
PETERS, R. A. Biochem. J. 30: 2206, 1936a.
Nature 138: 327, 1936b.
Chemisch Weekblad (Amsterdam) 34: no. 26, 1937a.
Biochem. J. 31: 2240, 1937b.
PETERS, R. A., H. RYDIN AND R. H. S. THOMPSON. Biochem. J. 29: 63, 1935.
PETERS, R. A. AND H. M. SINCLAIR. Biochem. J. 27: 1677, 1933.
PETERS, R. A. AND R. H. S. THOMPSON. Biochem. J. 28: 916, 1934.
PICK, E. P. Klin. Wchnschr. 16: 1481, 1937.
PLAUT, F. AND M. BÜLOW. Klin. Wchnschr. 13: 1744, 1934.
Ztschr. ges. Neurol. Psych. 153: 182, 1935.
Ztschr. ges. Neurol. Psych. 154: 481, 1936.
PUGH, C. E. M. AND J. H. QUASTEL. Biochem. J. 31: 286, 2306, 1937.
QUASTEL, J. H. AND M. STEPHENSON. Biochem. J. 19: 664, 1925.
QUASTEL, J. H., M. TENNENBAUM AND A. H. M. WHEATLEY. Biochem. J. 30: 1668, 1936.
QUASTEL, J. H. AND A. H. M. WHEATLEY. Biochem. J. 25: 117, 1931.
Biochem. J. 26: 732, 1932a.

Proc. Roy. Soc. B. **112**: 60, 1932b.
Biochem. J. **27**: 1753, 1933a.
Biochem. J. **27**: 1609, 1933b.
Biochem. J. **28**: 1521, 1934.
Biochem. J. **32**: 936, 1938.
QUASTEL, J. H. AND W. R. WOOLDRIDGE. Biochem. J. **22**: 689, 1928.
ROBERTSON, J. D. AND C. P. STEWART. Biochem. J. **26**: 65, 1932.
SCHMITT, F. O. Am. J. Physiol. **95**: 650, 1930.
SCHMITT, F. O. AND SKOW. Am. J. Physiol. **105**: 87; **106**: 44, 1933.
SCHNEIDER, D. AND M. SCHNEIDER. Arch. f. Exper. Path. **175**: 606, 1934.
SHERIF, M. A. F. J. Pharmacol. Exper. Therap. **28**: 11, 1930.
SHERIF, M. A. F. AND E. G. HOLMES. Biochem. J. **24**: *400, 1930.*
SHERMAN, W. C. AND C. A. ELVEJHEM. Am. J. Physiol. **117**: *142, 1936a.*
Biochem. J. **30**: 785, 1936b.
SHILLING AND ADAMS. U. S. Nav. M. Bull. **31**: 112, 1933.
SILBERSCHMIDT, R. Schweiz. Med. Wchnschr. **66**: 977, 1936.
SIMPSON, H. N. AND A. J. DERBYSHIRE. Am. J. Physiol. **109**: 99, 1934.
SINCLAIR, H. M. Biochem. J. **27**: 1927, 1933.
SYM, E., R. NILSSON AND H. V. EULER. Ztschr. physiol. chem. **190**: 228, 1930.
SZENT-GYORGYI, A. Biochem. Ztschr. **240**: 456, 1931.
TAKAHASHI, K. AND L. ASHER. Biochem. Ztschr. **154**: 444, 1925.
TOENNIESSEN, E. AND E. BRINKMAN. Ztschr. physiol. chem. **187**: 137, 1930.
TORRÈS, I. Biochem. Ztschr. **280**: 114, 1935.
VERNON, H. M. J. Physiol. **42**: 402; **43**: 96, 1911.
WACHHOLDER, K., K. ANDERS AND K. UHLENBROOK. Ztschr. physiol. chem. **233**: 184, 1935.
WARBURG, O. Biochem. Ztschr. **142**: 317, 1923.
Biochem. Ztschr. **152**: 309, 1924.
Metabolism of tumours. Constable & Co., 1930.
WEIL-MALHERBE, H. Biochem. J. **30**: 665, 1936.
Biochem. J. **31**: 2202, 1937a.
Biochem. J. **31**: 299, 1937b.
Biochem. J. **32**: 1033, 1938.
WESTENBRINK AND POLAK. Rec. Trav. Chim. Pays-Bas. **244**: 265, 1937.
WINTERSTEIN, H. Ztschr. physiol. chem. **120**: 1, 1922.
Die Narkose. J. Springer & Co., 1926.
Handbuch d. normalen und path. physiologie **9**: 246, 1932.
Pflüger's Arch. **235**: *377, 1935.*
Advances in Modern Biology **5**: no. 6, (Moscow), 1936.
WOHLGEMUTH, J. Biochem. Ztschr. **264**: 371, 1933.
WOLFF, H. G. Physiol. Rev. **16**: 545, 1936.
WORTIS, S. B. Arch. Neurol. Psych. **33**: 1022, 1935.
New York State J. Med. **38**: no. 14, 1938.
WORTIS, H., S. B. WORTIS AND F. I. MARSH. Arch. Neurol. Psych. **39**: 1055, 1938.
YAMAKITA, M. Tohoku J. Exper. Med. **3**: 414, 496, 538, 556, 1922.
YOUNG, L. Proc. Soc. Exper. Biol. Med. **35**: 507, 1936.
J. Biol. Chem. **120**: 659, 1937.
ZIMMERMAN, COWGILL AND FOX. Arch. Neurol. Psych. **37**: 286, 1937.

CELLULAR OXIDATION SYSTEMS

E. S. GUZMAN BARRON

Lasker Foundation for Medical Research and Department of Medicine, the University of Chicago

Combustion of foodstuffs through oxygen was Lavoisier's (207) conception of respiration. It is still ours, and for this reason the term *oxidation* has been retained in this review. Experiments since Lavoisier's time have discovered the intermediate steps between foodstuff and oxygen. Foodstuffs, which *in vitro* are oxidized only by powerful oxidants or under conditions which do not exist in biological systems, are oxidized in living cells at relatively low temperatures, at neutrality, through the action of mild oxidizing agents, the oxidizing enzymes. Thus cellular respiration, the consumption of oxygen accompanied by the production of CO_2 and H_2O, represents the integral of a number of partial oxidation-reductions, each of which has its peculiar mechanism. The work of the past ten years on the isolation of a number of oxidation enzymes and on the kinetic and energetic aspects of biological oxidations has given us a clearer understanding of the process of cellular respiration. It must be admitted that the fate of the simplest foodstuff, glucose, when it is used by living cells in the process of respiration, seems to preclude any possibility of a unified concept of cellular respiration. For glucose is directly oxidized by some cells; in others it is first split by glycolysis, the split products being partially oxidized in a few steps, all irreversible; in still others the split products of glycolysis are oxidized also in thermodynamically reversible steps introduced by enzymes; and, finally, in some cells glucose is oxidized to completion by the aid of a series of reversible oxidation-reduction systems of graded potential up to molecular oxygen, the energy being thus released gradually. In all these cases, however, respiration takes place by a transfer of electrons from the foodstuff through the oxidation catalysts to molecular oxygen. In the simplest cases the transfer is made by only one reversible system; in the more complicated cases the electron transfer is carried on step by step through a series of reversible oxidation-reduction systems, which regulate the release of energy, like locks in a canal, and prevent its sudden wasteful release. We may then conceive

of cellular respiration simply as the process by which the cell extracts energy from foodstuffs, this release of energy depending upon the transfer of electrons from the foodstuffs, first to systems capable of receiving them directly, and from them, by a series of oxidation-reduction systems, to atmospheric oxygen.

The conditions which make possible and regulate a determined reaction are two: energetic and kinetic. It is essential in the first place for the reaction to be thermodynamically possible. Afterwards we must search for the path it follows. The possibility of a reaction is determined by the knowledge of the equilibrium, concentrations of reactants, and products of the reaction. The data thus obtained are accurate, trustworthy. The path followed by the reaction, i.e., its mechanism, can be approached by studying the rate of reaction, its relation to the concentration of reagents, the influence of different substances which either inhibit or accelerate it, the effect of temperature, etc. The difficulties surrounding the kinetic aspect of the problem are obviously manifold and the answers obtained not so precise. But only through the understanding of the problem of equilibrium reactions (energetics) and that of the rate of these reactions (kinetics) can the problem of cellular respiration be approached with reasonable probability of success.

I shall, therefore, pass in review: first, studies related to the possibility of reactions, i.e., thermodynamic studies; second, studies related to the mechanism of reactions.

Oxidation-reduction systems of biological importance. Through the pioneering efforts of Clark and his collaborators, it is now possible to treat the subject of biological oxidations in a broad manner, for the fruitlessness of the discussions about whether biological oxidations are characterized by the addition of oxygen or the withdrawal of hydrogen was pointed out by Clark in 1923 (59). Clark at that time gave the following expression to a definition of oxidation that had had a long evolution: "The withdrawal of electrons from a substance with or without the addition of oxygen or elements analogous to oxygen; or as the withdrawal of electrons with or without the withdrawal of hydrogen or elements analogous to hydrogen."

If a reaction under investigation can be made to take place in a reversible galvanic cell, the maximum electromotive force of the cell may be measured by the potentiometric method and the free energy change of the reaction is then given by the equation,

$$\Delta F = -nFE,$$

where n is the number of equivalents of chemical change involved in the reaction equation, F is Faraday's constant (23,068 calories, when ΔF is to be expressed in calories), and E is the electromotive force of the cell.

When this simple method of determining free energies was introduced into biology by Gillespie (129) and Clark, it was found empirically that some thermodynamically reversible systems of biological importance behave unusually towards noble metal electrodes. And after years of toil it was recognized that besides those systems where their electron charges come immediately into equilibrium with those of a noble metal electrode, there are systems where the potentials can be obtained only on the addition of an electroactive substance. Furthermore, there are systems where the potentials are obtained only in the presence of an enzyme and an electroactive substance. The reason for this difference of behaviour is not known. Those belonging to the first group are the *electromotively active systems*; those belonging to the second group are the *sluggish systems*; those belonging to the third group are the *enzymatic-sluggish systems.*

Electromotively Active Systems. Electromotively active oxidation-reduction systems are those which, like $Fe^{++} \rightleftharpoons Fe^{+++} + e$, can exchange electrons directly with a noble metal electrode. As a rule, they are autoxidizable, i.e., readily oxidized by atmospheric oxygen. The free energy of these systems can be easily determined by the potentiometric method, the use of which was greatly stimulated among biologists by the studies of Clark and his co-workers, and by the publication of Michaelis' excellent monograph on the subject (227).

The free energies of these systems, i.e., the oxidation-reduction potentials, are determined directly by measuring with the potentiometer the E.M.F. given by two half cells, the standard and that of the system under study, and applying the equation

$$E_h = E_0 - \frac{RT}{nF} \ln \frac{[S_r]}{[S_0]} - \frac{RT}{nF} \ln K,$$

where E_h is the observed E.M.F. referred to the hydrogen electrode, E_0 is a constant involving all those constants which can be so combined, $[S_r]$ and $[S_0]$ the concentrations of total reductant and oxidant respectively, and K, a constant, function of H^+ and equilibrium constants.[1]

[1] For a derivation of this equation from the mass law see Clark and Cohen (65); for a thermodynamic derivation see Lewis and Randall (212).

Iron-porphyrin compounds.[2] The iron-porphyrin compounds occupy a unique position among the oxidation-reduction systems of biological importance, because on combining with nitrogenous compounds they give complexes possessing a variety of properties. Thus, when certain iron-porphyrins combine with a protein they form compounds (hemoglobin, myoglobin, erythrocruorin) which can combine with molecular oxygen reversibly, like the Werner complexes, with no electronic exchange; iron-porphyrins may combine with proteins and give non-autoxidizable sluggish oxidation-reduction systems (cytochrome *c*); finally, they may combine with proteins or other nitrogenous compounds giving autoxidizable, electroactive systems (hemochromogens, some of the cytochromes).

The properties of the first class of iron-porphyrin compounds (hemoglobin, myoglobin) will not be discussed in this review, although their respiratory function as oxygen storehouses is indeed of first importance in cellular respiration. Discussion will be limited to the last two groups, those able to act as electron mediators, which are directly concerned in oxygen utilization.

The classical paper of Keilin in 1925 "On cytochrome, a respiratory pigment, common to animals, yeast and higher plants" (166), and the elegant demonstration of Warburg and Negelein in 1928 (315) of the relative spectrum of their Atmungsferment, contributed to re-awaken the interest of investigators in iron-porphyrin compounds, the importance of which had been neglected since MacMunn's pioneer studies (see Anson and Mirsky's review (9)).

The universality of the distribution of iron-porphyrin compounds was recognized as early as 1886 (MacMunn (220)). They have been found in all aerobic bacteria (Yaoi and Tamiya (335); Tamiya and Yamagutchi (285), Yamagutchi (334); Frei, Riedmüller, and Almasy (110)); in plants (Kempner (178)); in lower animals (Roche (259)); in higher animals (Cohen and Elvehjem (68), Huszak (160)). They have been found even in organisms such as paramecium (Sato and

[2] To avoid confusion, the nomenclature used in this review will be as follows: *Iron-porphyrin* is used as a class name; *hemin* is used to designate the FeCl compounds of porphyrins, with a prefix designating the kind of porphyrin (blood hemin; spirographyshemin, pheohemin); *hemochromogen* is used for the nitrogenous coördination compounds of iron-porphyrin, the prefixes ferri- and ferro- indicating whether these compounds are oxidized or reduced—e.g., ferri- and ferro-nicotine hemochromogen. The word cytochrome has been retained because the nitrogenous compound bound to the iron-porphyrin nucleus is still unknown.

Tamiya (262)), whose respiration is insensitive to HCN (Gerard and Hyman (125)).

The property of iron-porphyrins and of their nitrogenous coördination compounds of possessing strong absorption spectrum bands, and that of some of these last compounds of combining reversibly with HCN and CO have been extensively used for their detection in living cells.

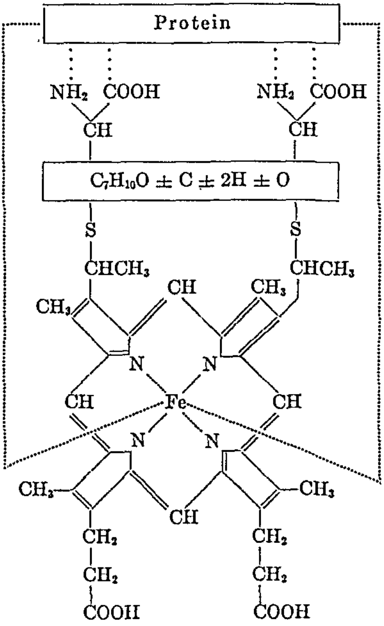
In table 1 are given representative examples of the position of the maximum absorption spectrum bands of hemochromogens, Keilin's cytochromes, as found in tissues and isolated cells. A comparison of these absorption bands to those obtained in solutions of iron-porphyrins and hemochromogens of known chemical constitution makes plausible the suggestion of Keilin (168) that the "cytochromes" found in living cells may be grouped in three classes: *a*, those with absorption bands around I, IV, and V; *b*, those with bands around II, IV, and V; and *c*, those with bands around III, IV, and V. The properties of these cellular hemochromogens have been described in detail by Keilin (168), Reid (256), and Shibata (268) in recent reviews. The chemical properties of cytochromes *a* and *b* are not yet known, although some work has been done by Roche and Benevent (262), who believe that their "heme *a*" as prepared from heart muscle has spectral properties similar to those of Keilin's cytochrome *a*, and by Yakushiji and Mori (332), who claim to have prepared natural and synthetic cytochrome *b*. The authors claim that both preparations are autoxidizable. More is known about cytochrome *c* as it has been prepared in pure form by Keilin and Hartree (172) and Theorell (289). Of these properties, the most important from the point of view of cellular respiration is that cytochrome *c* is not oxidized by measurable rate by atmospheric oxygen

TABLE 1

Position of the absorption spectrum bands of ferrohemochromogens present in cells (cytochromes)

	I	II	III	IV	V
Bee-wing muscles	6048	5665	5502	5210	4490; 4330; 4170
Guinea pig; heart muscle	6045	5662	5500	5205	?
Brewer's yeast	5860	5650	5510	5250	
Baker's yeast	6030	5650	5500	5230	
B. coli	6285; 5900	5600		5300	
B. pasteurianus	6350; 5890	5680	5520	5270	
Proteus mirabilis	6310; 5950	5600		5300	

in neutral solutions nor does it combine with HCN or CO at the concentrations used for respiration inhibitions (Altschul and Hogness (5) maintain that cytochrome *c* combines with CO). Cytochrome *c* is rapidly oxidized by Keilin's "cytochrome oxidase" (Keilin and Hartree (173)) and some hemochromogens (Mori, Okunuki, and Yakushiji (235)). The chemical constitution of cytochrome *c* has been studied by Theorell (293). According to him cytochrome *c* is made up of a porphyrine polypeptide complex containing two atoms of sulphur. The sulphur-containing side chain is bound to the porphyrin through both S atoms as in a thio-ether linkage. The protein component is bound to the side chain through peptide linkages of one or more of its amino and carboxyl groups; it is also bound to the Fe atom.



Heavy metals (Cu, Fe) combine with CO reversibly.

$$Fe^{++} + CO \rightleftharpoons Fe^{++}\,CO$$

Warburg (302) found that the respiration of yeast was inhibited by CO reversibly. During this process, with the assumption that the inhibition was due to the formation of a Fe^{++} CO compound, and that the remaining Fe^{++} compound is immediately transformed into Fe^{+++} compound by O_2, the ratio $\frac{Fe^{+++}}{Fe^{++}CO}$ depends on the $CO:O_2$ ratio according to equation

$$\frac{Fe^{+++}}{Fe^{++}CO} \times \frac{(CO)}{(O_2)} = K.$$

The value of K was determined by measuring the respiration at different ratios of $CO:O_2$, by use of the equation

$$K = \frac{r}{\left(\frac{V}{v} - 1\right)}$$

where V is the respiration in air; v, respiration in $CO:O_2$; and r, the ratio $CO:O_2$. The value of K in yeast was found to be 9.

Of the heavy metal compounds, only the CO iron compounds are sensitive to light. Warburg found that the CO inhibition of yeast respiration disappeared on illumination. By irradiating a $COFe^{++}$ compound by monochromatic light of different wave lengths and determining the degree of dissociation of this compound, a photochemical spectrum of the $COFe^{++}$ will be obtained. Warburg and his co-workers started by establishing in a number of iron-porphyrins the validity of the Einstein law of photochemical equivalence:

$$Fe^{++}CO + 1\,h\,v = Fe^{++} + CO,$$

where h is Planck's constant and v the frequency of the wave sent out. Since the concentration of the hemochromogen combining reversibly with CO is extremely small: less than 4×10^{-7} (Warburg and Negelein (316)), more than 3×10^{-8} g. Fe per gram of dry substance (Warburg and Kubowitz (313)), Warburg and co-workers therefore obtained the photochemical absorption spectrum of their "Sauerstoffübertragende Ferment der Atmung" by irradiating the cell suspension kept in a gas

phase of $CO:O_2$ with monochromatic light of different wave lengths (303).

The discovery of Anson and Mirsky (7) that hemochromogens are reversible compounds of iron-porphyrin and nitrogenous substances,

$$Fe^{+++} + 2\,N \rightleftharpoons Fe^{+++}N_2$$
$$Fe^{++} + 2\,N \rightleftharpoons Fe^{++}N_2$$

where N represents a nitrogenous substance, has been of first importance for the understanding of the properties of these substances. Ferri- and ferro-porphyrins combine as a coördination compound (Pauling and Coryel (248)) with 2 molecules of a nitrogenous substance, Hill (153), Langenbeck (206), Hogness et al. (156)) their equilibrium constants depending on the nature of the nitrogenous substance, hydrogen ion concentration, and temperature; furthermore, the coördination linkage may be made with two different nitrogenous compounds (Anson and Mirsky (8), Drabkin (88)). This great affinity of iron-porphyrins for nitrogenous compounds favors the opinion that they must always be present in living cells as hemochromogens; it also admits the possibility of rapid change from one hemochromogen into another through a change in the concentration of the nitrogenous substances and other factors which determine the value of the equilibrium constants.

Very little is known about the constitution of Keilin's cytochrome oxidase (indophenol oxidase). The enzyme is so intimately bound to the insoluble material of the cell that it becomes difficult to apply the ordinary methods of purification and isolation. It acts as a catalyst for the oxidation of cytochrome *c*, its catalytic effect being inhibited by HCN, H_2S, NaN_3, and CO (Keilin and Hartree (172)). Because of these inhibitions the belief has been widespread in the identity of Keilin's cytochrome oxidase and Warburg's "Sauerstoffübertragende Ferment der Atmung." It has been suggested that the enzyme may be a protein combined to cytochrome *a* or one of its components. Keilin and Hartree (173) present now strong arguments against this opinion and offer two other possibilities: the enzyme may be 1, an iron-protein compound devoid of porphyrin; 2, a copper-protein compound. The problem as to the nature of this enzyme remains open for further investigation.

Conant and his collaborators (71, 72) were the first to demonstrate that both the system ferrohemin-ferrihemin and the system ferrihemochromogen-ferrohemochromogen are electromotively active reversible oxidation-reduction systems, a one-electron transfer being involved in

the process. For blood hemin (26) the potential of the system at 30° is obtained from the equation

$$E'_h = E'_0 - 0.06 \log \frac{[Fe^{++}hemin]}{[Fe^{+++}hemin]} - 0.06 \log \frac{1}{H^+}$$

Where E_0 (the normal potential of the system at pH 0) in phosphate buffer was calculated to be +0.306 volt, and in borate buffer, +0.263 volt. The potentials of hemin became more positive on addition of a nitrogenous compound, the rise in potential being in inverse relation to the affinity of the nitrogenous compound for hemin. It was also found that when the affinity of the nitrogenous compound for hemin was great, as in the case of cyanide hemochromogen, the potential was independent of the hydrogen ion concentration; when the affinity was low, as in nicotine, α-picoline, and pyridine hemochromogens, the E_h values increased, as in hemin, by 60 millivolts per pH unit. The values of the potentials of spirographis hemin and some of its hemochromogens (Barron (27)) are more positive than those of blood hemin and its hemochromogens. All these hemochromogens are autoxidizable. Clark and his co-workers (67) have given a clear quantitative treatment of the empirical findings. By introducing the dissociation constants of the nitrogenous metallo-porphyrin compounds into the electrode equations, they have shown that if the affinity of the nitrogenous compound for the oxidized and reduced metallo-porphyrin is the same, there will be no change of potential on addition of the nitrogenous base. If the potential becomes more positive it means that the reductant forms the more stable compound; if the oxidant associated more strongly than the reductant, the trend of potential will be in the negative direction. Clark's treatment becomes of essential biological importance because of its possible extension to those numerous oxidation-reduction systems where the active nucleus (alloxazins, pyridinium nucleotides, and possibly thiamin) combines reversibly with nitrogenous substances.

The potentials of cytochromes will be discussed here only because of convenience, for some of these systems seem to act not as electroactive but as sluggish systems. At least, this is so in the case of cytochrome *c*, where attempts to titrate it according to Clark's methods for electroactive systems have failed (Green (133)). The potentials of the three cytochromes, *a*, *b*, and *c*, have recently been given in a short note by Ball (17). His values for cytochrome *c* are in fair agreement with those previously found by Coolidge (73) and Wurmser and Filitti-Wurmser (328). (These values for the potentials of cytochrome *c* have been

confirmed by Stotz et al. (280).) In table 2 are given the potentials (E'_0 values) at pH 7.0 of the cytochromes and of the iron-porphyrins and hemochromogens. One active nucleus, iron-porphyrin, because it combines reversibly with a number of nitrogenous substances, can alter its free energy so widely as to give the clearest example of a series of oxidation-reduction systems of graded potentials.

Alloxazins. (Two reviews have recently been published by Theorell (292) and Wagner-Jauregg (300).) The progress of our knowledge of the chemical constitution and properties of alloxazin has developed

TABLE 2

Oxidation-reduction potentials of hemins and hemochromogens

E'_0 values at pH 7.0, temp. 30°

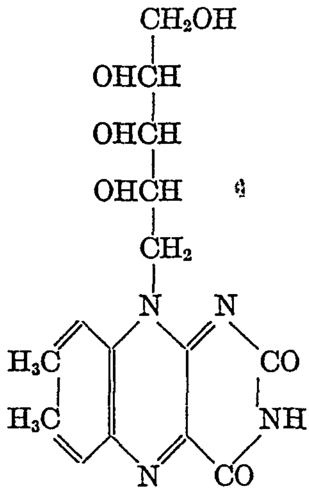
SYSTEM	E'_0
	volts
Blood hemin (phosphate)	−0.110
Nicotine hemochromogen	+0.184
Pyridine hemochromogen	+0.172
α-Picoline hemochromogen	+0.136
Histidine hemochromogen	−0.106
Globin hemochromogen	−0.098
Cyanide hemochromogen (pH 8.2)*	−0.183
Spirographis hemin	−0.077
α-Picoline spirographis hemochromogen	+0.155
Pilocarpine spirographis hemochromogen	−0.024
Cyanide spirographis hemochromogen (pH 8.2)*	−0.113
Cytochrome a pH 7.4	+0.290
Cytochrome b pH 7.4	−0.040
Cytochrome c pH 7.4*	+0.255

* Potentials independent of pH.

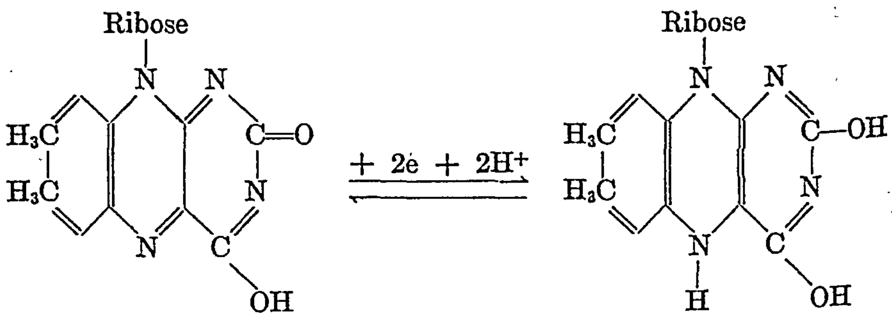
with extraordinary speed. Announced in 1932 as a new respiration enzyme, Warburg and Christian's (305) "gelbe Oxydations Ferment" in less than five years had its chemical constitution established, and but for its protein fraction we could have said the "yellow ferment" was synthetically prepared (Kuhn, Rudy, and Weygand (198)).

The alloxazins are widely distributed in nature, being present as riboflavin and its derivatives in animal tissues (Györgyi, Kuhn and Wagner-Jauregg (143), Euler and Adler (96)), in plants (Kuhn, Wagner-Jauregg and Kaltschmidt (200)), and in yeast and bacteria (Warburg and Christian (306)).

Riboflavin (6,7-dimethyl-9-d-ribityl-isoalloxazin) is a derivative of the hypothetical isoalloxazin, the yellow color of the pigment being due to the ring system of isoalloxazin. Kuhn and Wagner-Jauregg (199) were the first to show that the pigment was reversibly oxidized and reduced (although Warburg had previously found this property in his Yellow Ferment) and that in acid solutions the reduction of the

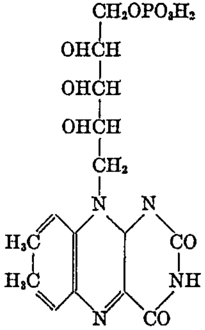


pigment proceeded through the intermediate formation of a semiquinoid radical. Riboflavin, as well as alloxazins, is an electroactive oxidation-reduction system. (The potentials of riboflavin and their derivatives have been measured by Klemperer, Bessy and Hastings (185), Kuhn and Moruzzi (197), Kuhn and Boulanger (195), Michaelis, Schubert, and Smythe (233), Stare (272), Stern (274).) Michaelis and his co-workers have found that the maximum amount of semiquinone formed at pH values of physiological significance is about 10 per cent. The reversible oxidation-reduction may be represented:

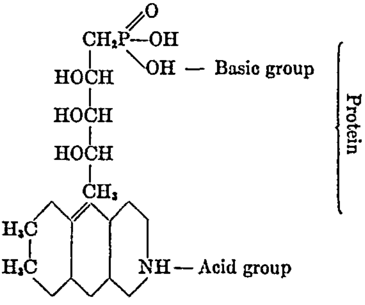


(For the structure of alloxazins in their various forms of oxidation-reduction and ionization, see Michaelis, Schubert, and Smythe (232).)

Riboflavin can be phosphorylated enzymatically by animal tissues (intestine) under the action of phosphatase (Rudy (261)), giving phosphoriboflavin:



Phosphorylation is also brought about by the adrenal cortex (Verzar, Hübner, and Lastz (297)). Phosphoriboflavin on combining with a protein gives phosphoriboflavin protein, with a molecular weight of 73,000 on the assumption that the compound contains one molecule of riboflavin. Kekwick and Pedersen (177) from measurements of the *sedimentation velocity and diffusion constant give 80,000 as the molecular* weight. The combination of flavin with protein is a reversible one as long as the protein component is not denatured. Theorell (291) and Kuhn et al. (195) have sggested that the basic protein group combines with the phosphoric acid group and the acid protein group with the basic imino group of riboflavin.



Warburg and Christian (310–311) have isolated from d-amino acid oxidase an alloxazin-adenine-dinucleotide which is formed by the union of riboflavin phosphoric acid and adenilic acid:

$$C_{17}H_{21}N_4PO_9 + C_{10}H_{14}N_5PO_7 - H_2O = C_{27}H_{33}N_9P_2O_{15}$$

This alloxazin has been found in all animal tissues and yeast, and like the other alloxazin is an autoxidizable, reversible oxidation-reduction system; it is also, presumably, an electroactive system. Ball (18), working in Warburg's laboratory, has isolated from milk xanthine oxidase, another alloxazin-adenine-dinucleotide. Corran and Green (74) have reported the isolation from milk of a new phosphoriboflavin-protein; whether this milk flavin-protein is part of the prosthetic group of xanthine oxidase or is a new alloxazin compound has not yet been established.

As hemin on combining with nitrogenous substances increases its potential toward more positive values, so, too, does phosphoriboflavin on combining with a protein (−0.061 volt at pH 7.0, 38°) (Kuhn and Boulanger (195)). Following the analogy, it is plausible to assume that different flavin-proteins may have different potentials.

Other oxidation-reduction systems. Besides these two systems whose rôle as catalysts of oxidations has been established, there have been found in biological fluids a number of substances which are electromotively active, and have been considered accessory respiration catalysts. (In table 3 are given the E'_0 values of these systems at pH 7.0.)

Michaelis two-step oxidation. (Michaelis has written three excellent reviews (228–230).) The discovery of Michaelis and Elema that pyocyanine (a phenazine dye) in acid solutions was reduced in two steps with the formation of an intermediate compound or semiquinone has attained considerable importance because of the ever-increasing number of systems being found in living cells which show semiquinone formation. First found only among cationic systems they were later also found among anionic systems after Preisler and Shaffer (252) reported the first example of anionic dye reducing in two steps in highly alkaline solutions. Suppose a substance R which can be reversibly oxidized to a substance $T(R \rightleftharpoons T + 2\ e)$ in two successive steps, each involving the loss of one electron:

$$R \rightleftharpoons S + e; S + e \rightleftharpoons T + e.$$

The system can exist in three different forms: R (reduced form), S (semi-oxidized form), and T (totally oxidized form). As the S form can

dismute reversibly: $2\ S \rightleftharpoons R + T$, the equilibrium k of the dismutation will be:

$$k = \frac{[R][T]}{[S]^2}$$

where k is Michaelis' dismutation constant; its reciprocal value $K = \frac{1}{k}$ is what he calls semiquinone formation constant. When a solution

TABLE 3

Oxidation-reduction potentials of electroactive systems present in biological fluids

E'_0 values at pH 7.0

SUBSTANCE	ORIGIN	E'_0	TEMP.	ELECTRON TRANSFER	REFERENCE
		volts	°C.		
Adrenalone	Animal	+0.489	30	2 by 2 (?)	Ball, Chen, and Clark (19)
Epinephrine	Animal	+0.389	30	2 by 2 (?)	Ibid.
Homogentisic acid	Animal	+0.260	30	2 by 2 (?)	Fishberg and Dolin (108)
Phoenicein	Mold	+0.047	20	2 by 2 (?)	Friedheim (113)
Juglone	Plant	+0.036	20	2 by 2 (?)	Ibid. (116)
Pigment from *Arion rufus*		+0.025	20	2 by 2 (?)	Ibid. (112)
Hallachrome	Animal	+0.022	20	1 by 1	Ibid. (114)
Pyocyanine	Bacteria	−0.034	30	1 by 1	Friedheim and Michaelis (117); Elema (89)
Hermidin	Plant	−0.030		2 by 2	Cannan (56)
Toxoflavin	Bacteria	−0.049		1 by 1	Stern (275)
Phosphoriboflavin-protein	Yeast	−0.061	38	1 by 1	Kuhn and Boulanger (195)
Chromodoris zebra pigment	Plant	−0.102	30	1 by 1	Preisler (251)
Chlororaphin	Bacteria	−0.115	30	1 by 1	Elema (90)
Lawsone	Plant	−0.139	20	1 by 1 (?)	Friedheim (116)
Phthiocol	Bacteria	−0.179	30	1 by 1	Ball (13); Hill (152)
Lapachol	Plant	−0.180	30	1 by 1	Ball (14) (16); Hill (152a)
Lomatiol	Plant	−0.184	30	1 by 1	Ball (14); Hill (152a)
Riboflavin	Animal and vegetable	−0.208	30	1 by 1	Michaelis, Schubert and Smythe (232)
Echinochrome	Animal	−0.221	30	2 by 2 (?)	Cannan (57)

containing only R is titrated with an oxidant the magnitude of k will determine how much of S can be formed *in maximo* during the titration. If k is very small the two steps will greatly overlap. If this constant is very large, at the beginning of the oxidative titration, only S will be formed, and T will arise only after practically all R has been converted into S. In this case each of the two steps of oxidation can be treated separately, as there is practically no overlapping. Accordingly k can be estimated from the curve obtained by experiments. From the titration curve E_1 (the difference of potential at 50 per cent of the titration

and that at 25 per cent), the index potential is determined by graphic interpolation. E_m (the normal potential of the system corresponds to Clark's E'_0) is always the potential at 50 per cent of titration; E_2 is the potential at 75 per cent of titration. In general

$$E_m - E_1 = E_2 - E_m = \frac{RT}{F} \ln k.$$

The following oxidation-reduction systems naturally occurring in living cells have been found to transfer electrons one by one: riboflavin and phosphoriboflavin protein, pyocyanine, chlororaphine, hallochrome, toxoflavin, pthiocol, lapachol, and lomatiol. The two-step oxidation of pthiocol in alkaline solutions was shown by Hill (152); that of lapacol was demonstrated by Ball (16) and Hill (152a). It is quite likely that other systems (for example lawsone, juglone, which are naphthoquinone derivatives) present in biological fluids oxidize step by step. This opinion is supported by Hill's (152a) recent paper. As will be seen later, similar two-step oxidation has been found in pyridine-nucleotides and possibly thiamine. The case of phosphoriboflavin-protein is interesting because it seems that radical formation is increased when it combines with diphosphopyridine-nucleotide ((Haas) (144)).

Sluggish Oxidation-Reduction Systems. Sluggish oxidation-reduction systems are, as a rule, non-autoxidizable, i.e., they are not oxidized by atmospheric oxygen at a measurable rate and within pH values found in physiologic conditions. They are easily oxidized by electromotively active substances, and easily reduced by a number of oxidizable substances. Although thermodynamically reversible, they are electromotively sluggish and their potentials cannot be satisfactorily measured but with the aid of electroactive mediators. The potentials can then be measured potentiometrically, colorimetrically or spectrophotometrically, provided the sluggish system or the electroactive mediator can be determined by those methods. In effect, if we assume that thermodynamic equilibrium is established between the sluggish system *sl* $((\mathrm{Ox})_{sl}^{+++} + e \rightleftharpoons (\mathrm{Red})_{sl}^{++})$ and the electroactive system *el* $((\mathrm{Ox})_{el}^{+++} + e \rightleftharpoons (\mathrm{Red})_{el}^{++})$, the equilibrium constant K for the reaction is:

$$K = \frac{(\mathrm{Red}_{sl})(\mathrm{Ox}_{el})}{(\mathrm{Ox}_{sl})(\mathrm{Red}_{el})}$$

Since in the equilibrium mixture the potentials must be the same for both substances:

$$E'_{0sl} - E'_{0el} = \frac{RT}{F} \ln K; \qquad E'_{0sl} = E'_{0el} - \frac{RT}{F} \ln K.$$

This electroactive sluggishness was, before its recognition, a source of confusion and the origin of numerous conflicting statements about their reversibility.

Thiols. In 1921 Hopkins succeeded in isolating a sulphydril compound, first from yeast, then from all tissues, to which he gave the name glutathione (glutamyl-cysteinyl-glycin) (Hopkins (157), Kendall (180)). This discovery produced intense interest because it was thought at the time that an oxidation catalyst had been isolated, since the system could be easily reduced by tissues and, it was claimed, easily oxidized by atmospheric oxygen. The non-autoxidation of the thiol systems was demonstrated first for cysteine by Warburg and Sakuma (318); for glutathione by Meldrum and Dixon (222). Both cysteine and glutathione are easily oxidized by copper and hemochromogens (Krebs (188), Lyman and Barron (219)).

The failure of all the attempts made to measure the oxidation-reduction potential of thiols by direct potentiometric methods (Dixon and Quastel (86), Dixon (84), Michaelis and Flexner (231), Green (131), Ghosh and Ganguli (127)) is due partly to the sluggishness and partly to the formation of complex compounds between the electrode and the thiol (Barron, Flexner, and Michaelis (30)). Recently, measurements made under conditions described as thermodynamically reversible have been published, although the values found (E'_0 at pH 7.0 for the reaction 2 cysteine $\rightleftharpoons$ cystine + 2 H^+ + 2 e, +0.13 volt (Williams and Drissen (323)); −0.34 volt (Ghosh, Raychandri, and Ganguli (128); −0.23 volt (Fruton and Clarke (119))) differ too much among each other, as well as from those obtained from thermal data (−0.40 volt (Borsook, Ellis, and Huffman (45))) to be considered as due to experimental errors.

The potential of thiols ((S—S) + 2 e + 2 H^+ $\rightleftharpoons$ 2(SH)) at a given pH may be written:

$$E_h = E'_0 - \frac{RT}{nF} \ln \frac{(SH)^2}{(S-S)}$$

If the sum of the concentrations of (SH) and (S—S) is rewritten in the form of a degree of oxidation (x) of (SH):

$$E_h = E'_0 + \frac{RT}{nF} \ln 2\, SH \frac{(1-x)^2}{x}$$

The potential observed is thus a function not only of the degree of oxidation but also of the concentration.

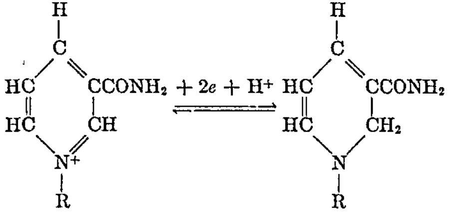
Ascorbic Acid. Another system of great reducing power is ascorbic acid (see review by King (181)), which, like glutathione, is present in biological fluids mostly in its reduced form. Non-autoxidizable in neutral solutions, it is easily oxidized by metallic catalysts, such as copper, and by hemochromogens (Barron, DeMeio, and Klemperer (29)). Protected in animal fluids from oxidation by glutathione and proteins (Barron, Barron, and Klemperer (28)) it is oxidized in certain vegetable fluids, the oxidation being due to the absence of protective power and the presence of oxidizing catalysts which are considered by some investigators as specific enzymes (see Tauber's review (287)). Some of these catalysts ("ascorbic acid oxidase") have an iron-porphyrin as active nucleus, some of them may resemble copper protein complexes (Stotz et al. (278)). In small concentrations, glutathione protects the oxidation of ascorbic acid by combining with copper present in the solute; in large concentrations, it prevents the oxidation of ascorbic acid (Borsook et al. (44)) by virtue of its more negative potential; under such conditions (ratio of glutathione: ascorbic acid, 100:1) glutathione may also reduce dehydroascorbic acid. Much has been written about the reversibility of ascorbic acid oxidation catalyzed by "ascorbic acid oxidase." The chemical reversibility of the system is conditioned exclusively by the hydrogen ion concentration, as shown in studies on the oxidation of ascorbic acid by copper, and its reduction by H_2S, the degree of reversibility depending on the degree of stability of the reversible oxidation product.

Characteristic proof of the sluggishness of this system is found in the contradictory statements about its thermodynamic reversibility, some investigators maintaining that the system is thermodynamically irreversible (Laki (201), Karrer, Schwarzenbach, and Schöpp (163), Green (132)), others maintaining that it is reversible (Georgescu (121), Borsook and Keighley (46), Wurmser and de Loureiro (330), Fruton (118)). By using the technique required for the determination of the potential of this group of compounds, that is, the addition of an electroactive mediator, Ball (14) restudied the subject and confirmed the reversibility of the system.

Pyridine-nucleotides. (Codehydrogenases, coenzymes, coferments.) (Myrback has written a review of diphosphopyridine-nucleotide (cozymase). See also reviews by Euler (94, 95) and Warburg (304).)

We owe our knowledge of the chemical constitution of pyridine nucleotides to the laboratories of Euler, Warburg, and Karrer. In 1934

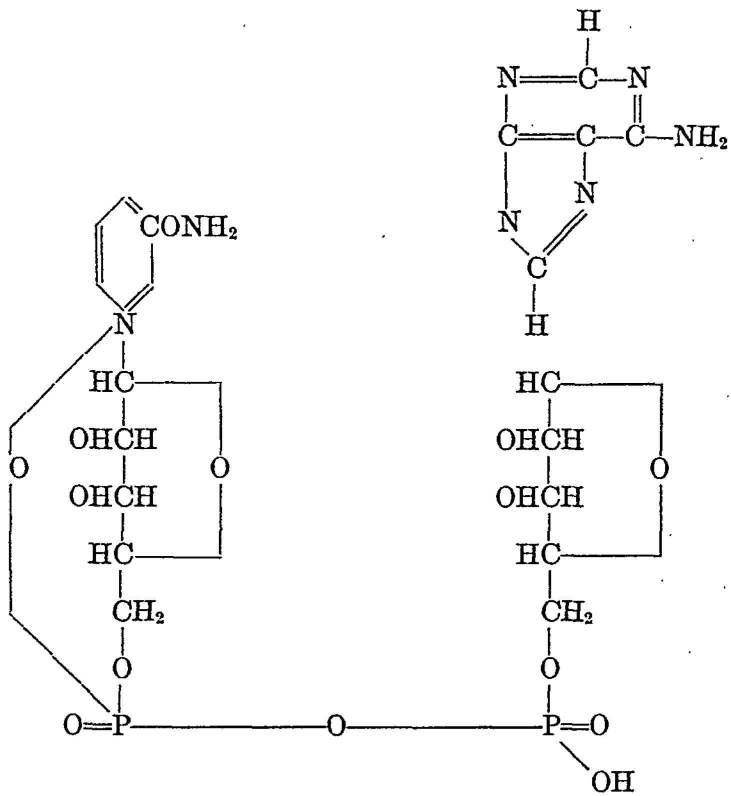
Warburg and Christian (308) found that their "coferment II" (one of the systems taking part in the enzymatic oxidation of hexose monophosphoric acid) contained a nicotinic acid amide group. Nicotinic acid amide is not reduced by $Na_2S_2O_4$; it is irreversibly reduced by Pt-H_2 to a piperidine compound with the uptake of six atoms of hydrogen. The addition of a radical to the nitrogen of nicotinic acid amide (for example, iodo-methyl-nicotinic acid prepared by Karrer and Warburg (165)) makes the compound reversibly reduced by $Na_2S_2O_4$ with the liberation of acid.



This is the active nucleus of the pyridinium derivatives of biological significance.

The oxidation-reduction process can be easily followed spectrophotometrically because the oxidized compound has (a characteristic property of these pyridinium derivatives) a strong absorption band around 2600 Å which on reduction diminishes, being replaced by another at 3400 Å, and because the reduced compound shows strong fluorescence under a white light (Warburg and Christian (308)).

The two pyridine nucleotides so far isolated, "cozymase" and "coenzyme II" contain besides the active nucleus, nicotinic acid amide, a purine, adenine, two pentoses, α-ribose, and phosphoric acids. The phosphoric acid is linked to ribose in the 5 position according to Euler and Karrer (101). "Cozymase" contains two phosphoric acid groups being a diphosphopyridine nucleotide. Its structural formula—tentatively suggested by Euler and Schlenck (102)—is



Euler and Schlenk (102) and Ochoa (243) have described simple methods for the preparation of diphosphopyridine nucleotide from yeast and muscle.

Coenzyme II is a triphosphopyridine nucleotide. Warburg, Christian, and Griese (312) prepared it from horse red cells. It can now be prepared from diphosphopyridine nucleotide by phosphorylation with $POCl_3$ (Schlenk (263)) or by enzymic phosphorylation (Euler and Adler (97), Vestin (298)).

Pyridine nucleotides are two-step oxidation systems; thus the methyl iodide of nicotinic acid amide when reduced with $Na_2S_2O_4$ in slightly alkaline solutions (pH 8.0) gives an intermediate compound, a free radical, of orange-yellow color (Karrer and Benz (161)). An intermediate reduction compound has also been found in the reduction of diphosphopyridine nucleotide (Adler, Hellström and Euler (2)) which is considered to be "monohydrocozymase" by Hellström (151).

In the reduced state and at physiological pH values none of these compounds is oxidized at a measurable rate by atmospheric oxygen. They are oxidized with great speed by phosphoriboflavin protein (Haas

(144)) and other electroactive systems of suitable potential (methylene blue, possibly hemochromogens). In the cell it seems that the reduced phosphopyridine nucleotides are oxidized by an enzyme discovered independently by Dewan and Green (77) and Adler, Euler, and Hellström (1). The enzyme has been found in animal tissues, yeast, and bacteria (Green and Dewan (137)). Like cytochrome oxidase it strongly adheres to the solid structures of the cell; the enzyme seems to contain no flavoprotein; its chemical constitution is still unknown.

The electroactive sluggishness of pyridine nucleotides is demonstrated by the failure of the numerous attempts made to determine by direct potentiometric titration the potentials of synthetic nicotinic acid amide compounds (Karrer and co-workers (162)) and of diphosphopyridine nucleotide (Hellström (151)). The potentials of these systems may be measured electrometrically by the aid of electroactive systems or by equilibration with other reversible systems. Use of the first method may necessitate the preparation of more stable oxidation-reduction dyes of quite negative potentials, because these systems when reduced have, as a rule, strong reducing power. (Thus the orthoderivatives of dihydronicotinic acid, amide: -n-methyl-o-n-ethyl-o and n-propyl-o-n-butyl-o- gave E_h values around -0.48 volt at pH 9, which would give roughly an E'_0 of about -0.4 volt.) Use of the second method has been made by Clark (64) for calculating the potentials of diphosphopyridine nucleotide (combined with the activating protein for lactic acid oxidation?) from Euler et al. (100) measurements of the equilibrium constant k for the reaction

$$\text{Pyruvate} + \text{D.P.N.} \rightleftharpoons \text{Lactate} + \text{D.P.NH}_2$$

At pH 9 (25°C.) the E'_0 of diphosphopyridine-nucleotide would be -0.37 volt.[3] It seems therefore probable that all these pyridine-nucleotides may have quite negative potentials, their values changing slightly on changing the nature of the group attached to the quaternary N of nicotinic acid amide (Karrer, Schwarzenbach, Benz and Sohmssen (162), Karrer and Stare (164), Schlenk, Hellström, and Euler (264)).

As iron-porphyrins combine reversibly with nitrogenous substances—proteins among them—so pyridine nucleotide (D.P.N.) and its reduc-

[3] Schlenk, Hellström, and Euler (264) have made similar calculations. Unfortunately they chose for these calculations E_0 values for ethyl alcohol $\rightleftharpoons$ acetaldehyde which were given by Wurmser and Filitti-Wurmser (327) only as tentative values, and E_0 values for malic acid $\rightleftharpoons$ oxaloacetic acid (Laki (203)) which were obtained only at one concentration of reactants. The E'_0 values for diphosphopyridine nucleotides given by them are about 32 m.v. more negative than those calculated by Clark.

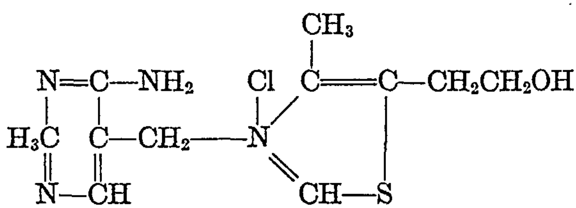
tion product (D.P.NH$_2$) combine reversibly with proteins (Negelein and Gerischer (240), Negelein and Wulff (242)):

$$\text{D.P.N.} + \text{protein} \rightleftharpoons \text{P.N.} - \text{protein} \tag{1}$$
$$\text{D.P.NH}_2 + \text{protein} \rightleftharpoons \text{D.P.NH}_2 - \text{protein} \tag{2}$$

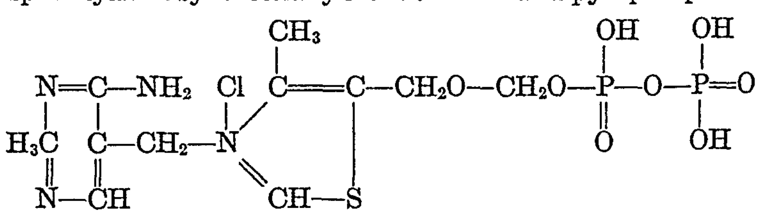
When diphosphopyridine nucleotide combines with the protein for the oxidation of ethyl alcohol, the equilibrium constants of reactions (1) and (2) have different values: $k = 9 \times 10^{-5}$ for reaction (1); 3×10^{-5} for reaction (2) (Negelein and Wulff (242)). When triphosphopyridine-nucleotide combines with the protein for the oxidation of hexosemonophosphate, the equilibrium constants for reactions (1) and (2) are the same: $k = 1 \times 10^{-5}$ (Negelein and Haas (241)).

Will the potentials of pyridine-nucleotides, like those of iron-porphyrins, change on combining reversibly with the proteins? Will in such a case Clark's conception of the relation of potential to the equilibrium constants of the oxidized and reduced system be applicable? These are problems for future research.

Thiamin (*vitamin* B_1), a thiazol derivative containing a primary alcohol and a quaternary pyrimidine ring, the structural formula of which was elucidated by Williams and his co-workers (324) contains also a quaternary nitrogen atom in a position comparable to its position in phosphopyridine-nucleotides:



Thiamin on phosphorylation takes up one or two phosphoric acid groups; thus is formed diphosphothiamin, "cocarboxylase," the constitution of which was established by Lohman and Schuster (217). The phosphorylation is performed enzymatically by phosphorylation enzymes (Tauber (286), Euler and Vestin (103), Lipschitz, Potter and Elvehjem (216)). Tauber (288) has recently succeeded in preparing diphosphothyamin synthetically from thiamin and pyrophosphoric acid.



Thiamin may be reduced with $Na_2S_2O_4$ or Pt-H_2 as Lipmann (215) has shown. Diphosphothiamin is also reduced with $Na_2S_2O_4$ (Barron and Lyman[4]). Both of these reduced substances are strong reducing agents and non-autoxidizable, i.e., not oxidized by atmospheric oxygen; they are, however, easily oxidized by the catalytic action of blood hemin and hemochromogens with an uptake of one atom of oxygen per mole of compound. These experiments and the close structural relation between thiamin and pyridine nucleotides made plausible the assumption that thiamin and diphosphothiamin are sluggish reversible oxidation-reduction systems. Unfortunately neither the reduced products nor the reoxidized compounds are physiologically active. It is quite possible that during the reduction process with $Na_2S_2O_4$ the thiamin molecule is split into pyrimidine and thiazol, as neutral sulfite solutions split thiamin at room temperature, a reaction which was discovered by Williams and was the starting point for his brilliant work on the synthesis of the vitamin.

Enzymatic-sluggish oxidation-reduction systems. When an indifferent electrode, gold or bright platinum, is immersed in an oxygen-free buffered solution containing a mixture of lactate and its oxidation product, pyruvate, no potential indicative of electron transfer is obtained at the electrode. The addition of α-hydroxyoxidase derived from gonococci gives to this system, lactate-pyruvate, a slight tendency to transfer electrons to the electrode; i.e., an erratic potential is obtained which drifts slowly towards negative values without attainment of equilibrium. But when an electromotively active system (for example, a dye) previously shown to be only partially reduced by this system, is added, there is obtained at the end of one to two hours a stable, reproducible potential. Although the potential is due directly to an electron transfer from the electroactive system, this system acts simply as a mediator, for its reduction has been brought about by the system lactate-enzyme-pyruvate. The values obtained may therefore be taken as representing the potential of this system. The potentials of these reversible systems thus require the mediation of two factors: the enzyme, which acts as a perfect catalyst, and the electroactive system, which is used to measure the potential of the system. These potentials may therefore be measured either electrometrically or colorimetrically.

The first of these systems to be discovered was the succinate-fumarate system. The reason for this early discovery can be seen in the wide use of methylene blue as an indicator of oxidation, which followed

[4] Unpublished experiments.

Ehrlich's pioneer work on "Das Sauerstoffbedürfnis des Organismus" published in 1883. It happens that the potential of this system lies around that of methylene blue. Wishart (325) discovered the reversibility; Quastel and Whetham (253) measured the equilibrium constant, and Borsook and Schott (47) demonstrated that the values for the free energy of this system, as obtained colorimetrically (Quastel and Whetham (253), Thunberg (294) or electrometrically, Lehmann (208), Borsook and Schott (47)) agreed remarkably well with those obtained with the help of the third law of thermodynamics.

Since then a number of enzymatic sluggish oxidation-reduction systems have been discovered covering a wide range of potentials, from the hydrogen potential (the system $HCO_2^- + H_2O \rightleftharpoons HCO_3^- + H_2$) studied by Woods (326) up to the methylene blue potential (the succinate ⇌ fumarate system).

TABLE 4

Potentials of enzymatic sluggish oxidation-reduction systems; E'_0 values at pH 7.0

SYSTEM	E'_0	TEMP.	REFERENCE
	volts	*°C.*	
Succinate ⇌ fumarate	0.000	38	Lehmann (208); Borsook and Schott (47)
d(−)Alanine ⇌ ammonium pyruvate	−0.048	37	Wurmser and Filitti-Wurmser (329)
Malate ⇌ oxaloacetate	−0.169	37	Laki (203)
Lactate ⇌ pyruvate	−0.180	35	Barron and Hastings (33); Wurmser and Mayer-Reich (331)
Ethyl alcohol ⇌ acetaldehyde	−0.190	30	Calculated from Adler's equilibrium measurements (3)
Isopropyl alcohol ⇌ acetone	−0.251	30	Wurmser and Filitti-Wurmser (327)
β-Hydroxybutyrate ⇌ aceto-acetate	−0.282	38	Green, Dewan and Leloir (138)
Xanthine ⇌ uric acid	−0.361	30	Green (134); Filitti (106)
Hypoxanthine ⇌ xanthine	−0.371	30	Ibid; ibid.
Formate ⇌ $H_2 + CO_2$	−0.420	30	Woods (326)

The system ethyl alcohol ⇌ acetaldehyde (important because it is to alcoholic fermentation what the system lactic acid ⇌ pyruvic acid is to lactic acid fermentation) was studied by Lehman (209); as calculated from Lehmann's paper the E'_0 of this system at pH 7.0 is −0.063 volt; the value of −0.190 volt given in table 4 was obtained by calculation from the equilibrium measurements reported by Adler and Sreenivasaya (3). The equilibrium constant K for the reactions alcohol ⇌ acetaldehyde and diphosphopyridine nucleotide (D.P.N.) ⇌ dihydrodiphosphopyridine nucleotide (D.P.NH$_2$),

$$K = \frac{[\text{D.P.N.}]\,[CH_3CHOH]}{[\text{D.P.NH}_2]\,[CH_3COH]}$$

was determined at various pH values.

The potential of alcohol $\rightleftharpoons$ acetaldehyde is:

$$E'_{0_{\text{alcohol}}} = E'_{0_{\text{D.P.N.}}} - \frac{RT}{2F} \ln K.$$

(The E'_0 values of diphosphopyridine nucleotide given by Clark (64) were used.) The difference between this value and that obtained by Lehmann is too great to be attributed to experimental error. To check the validity of both values, those calculated by Clark for diphosphopyridine nucleotide and those calculated here for alcohol $\rightleftharpoons$ acetaldehyde, one can determine the free energy ΔF of this reaction with data where no enzyme is concerned. Parks and Huffman (246) have calculated the free energy for the reaction

$$CH_3CH_2OH \text{ (gas)} = CH_3CHO \text{ (gas)} + H_2$$
$$\Delta F^\circ = 11{,}400 - 4.0\,T \ln T + 9.96\,T$$ [5]
$$\Delta F_{298^\circ} = 7586 \text{ calories}$$

For the reaction in aqueous solution, to calculate the molal free energy change, the vapor pressure data for acetaldehyde given by Bushmakin and Kuchinskaya (55) (99 mm. Hg per 1 M acetaldehyde) and that of alcohol given by Dobson (87) (3.65 mm. Hg) were used. As a result:

$$\Delta F^0_{298} = 7586 + RT \ln \frac{99}{3.65} = 9540 \text{ calories } (E_0 = +0.230 \text{ volt})$$

ΔF_{298° as calculated from the E_0 value (+0.223 volt) is 10280 calories. The agreement is satisfactory. Moreover, it gives greater validity to Clark's calculations of the oxidation-reduction potential of diphosphopyridine nucleotide.

The reaction α-glycerophosphate $\rightleftharpoons$ glyceraldehyde phosphate is also reversible (Meyerhof and Kiessling (226)). The equilibrium constant for the reaction,

$$\alpha\text{-glycerophosphate} + \text{D.P.N.} \rightleftharpoons \text{glyceraldehyde phosphate} + \text{D.P.NH}_2,$$

has been measured at 30° by Euler et al. (99). The E'_0 at pH 7.0 for the reaction would be −0.180 volt.

Free Energies and Rates of Reactions. It seems that a number of biologists still stress the importance of the heat of reaction and consider

[5] The value of 9.96 for I (integration constant of the free energy equation) is the average obtained from Bancroft and George's (20) data on the equilibrium of this reaction.

it the criterion for determining the direction of a chemical reaction. In the latter point, however, they are incorrect, because the factor which determines that a given process will take place, or at least *may* take place spontaneously, is not the heat evolved in the process but rather the free energy evolved. If the reaction proceeds at constant pressure

$$\Delta F = \Delta H - T\Delta S.$$

The free energy decrease $-\Delta F$ of the reaction is equal to the heat of the reaction $-\Delta$H only in case $\left(\frac{\delta(\Delta F)}{\delta T}\right)_p = 0$, where p = pressure. If the temperature coefficient of the free energy decrease of a reaction at constant pressure is negative, $-\Delta F > -\Delta H$; if it is positive, $-\Delta F < -\Delta H$. Thus a knowledge of ΔF is of great utility to the biologist. If ΔF has a large negative value, the process involved may take place spontaneously at constant temperature and pressure; if ΔF is positive for a process, there must be an increase of free energy in the system and the process will take place only through the application of work; if ΔF is zero, a state of equilibrium exists and no further change is to be expected. Constant concentrations other than standard are here assumed.

From a knowledge of ΔF for the process at one atmosphere the equilibrium constant K for the reaction can be calculated by means of the thermodynamic relationship

$$\Delta F^\circ{}_T = -RT \ln K.$$

Neglect of these fundamental considerations has led to a number of misconceptions. From quite an extensive bibliography let us quote only two examples: Wieland (327) reported the oxidation of hydroquinone by palladium, a reaction which requires so large a quantity of energy "that the probability of the process occurring in the direction specified falls in the same category as the probability that a kettle of water will boil when placed on ice" (Clark (63)). The reaction as stated by Wieland and presented as example of his "hydrogen activation" theory was proved by Gillespie and Liu (130) not to take place. Szent-Györgyi (282) observed that paraphenylene diamine was rapidly oxidized by washed muscle tissue in the presence of oxygen; that oxidation did not occur when methylene blue instead of oxygen was used as "hydrogen acceptor." He then concluded: "*Bei der Oxydation des Paraphenylenediamins durch das Muskelgewebe findet also keine Wasserstoffaktivierung statt.*" If Szent Györgyi had been in possession of the

free energy data of these two reversible systems (E'_0 at pH 7.0, temp. 30°, of the p. phenylenediamine $\rightleftharpoons$ quinonediimine system, +0.381 volt (Fieser (105)); of methylene blue, +0.011 volt (Clark, Cohen and Gibbs (66))) he would not have been put to the necessity of postulating that the oxidation of p-phenylene diamine was not performed through hydrogen activation.

Very valuable work, that of determining the free energies of substances of biological importance, is now being done at the California Institute of Technology by Borsook, Huffman, Parks and co-workers.

While ΔF enables us to predict the direction and extent of a reaction, it can tell us nothing regarding the time necessary for the attainment of a condition of equilibrium. "At one time the equation Reaction velocity $= \frac{\text{Chemical affinity}}{\text{Chemical resistance}}$ was used in a qualitative and descriptive way, but it is rather misleading, because there is no correlation whatever between chemical affinity, as measured by the maximum work, and speed of reaction. Affinity depends solely upon initial and final states. Velocity of reaction, on the other hand, depends upon the nature of some specific reactive state in which the molecules become capable of rearrangement" (Hinshelwood (154)). This statement *summarizes the position taken by students of chemical kinetics* whenever an attempt was made to correlate free energy with speed of reaction. However, some voices of protest were heard from time to time. Witness Conant's work in 1926 (70) on "the apparent reduction potential" of reducing substances capable of an irreversible reduction; LaMer and Temple's work in 1929 who reported that the rate of oxidation of hyroquinone under the catalytic influence of manganous salts was a function of the available free energy of the system. In 1930 (Barron and Hoffman, 34) it was found that the catalytic power of reversible dyes on cellular respiration was conditioned by two factors: the oxidation-reduction potential of the dye, and the permeability of the cell membrane; the speed of this catalysis was correlated to (1) the speed at which the dye is reduced by the cell, and (2) the speed at which the leuco-dye is oxidized by atmospheric oxygen. Such a correlation between *the free energy of the dyes (as determined by their potentials)* and their influence on cell respiration was naturally received with caution. However, a study of the rate of oxidation by atmospheric oxygen of reduced electroactive dyes showed the same relation between the free energy and the rate of oxidation (Barron (25)). Since then, numerous papers have been published from different laboratories,

confirming these observations (Stotz and Hastings (279), Chow and Kammerling (61), Chow (60), Hammett (146), Laki (202), Dimroth (81), Dewan and Green (78)).

The theories of reactions recently developed by Eyring (104), Rice (257, 258), La Mer (204) and Gerschinowitz (126), which make explicit use of an activated complex, show that the rate of a reaction is determined by a free energy change which is usually not the same as the free energy for the total reaction. They have shown that the rate of a chemical reaction is determined by the concentration of the reacting systems in a certain fraction of the total phase space that is available to the systems, multiplied by the velocity with which the systems are passing through this region. The configuration of the reacting molecules which corresponds to this phase space may be called the active complex. They postulate (see Gerschinowitz for derivation of equation) that the specific rate constant (k_1) of these reactions depends on the free energies of the activated systems:

$$k_1 = \exp \frac{-\Delta F^*}{RT} \frac{kT}{h}$$

where ΔF^* is the free energy of formation of the activated state, and k and h the Boltzmann and Planck constants, respectively. In general, ΔF^* is not the same as ΔF, the free energy change for the total reaction. In order to have the free energy change in such reactions equal to the free energy of formation of the activated complex, it would be necessary that the energy of formation of the activated state be equal to the energy of the reaction. (Three such cases have been reported: LaMer and Temple (205), Dimroth (81), and Barron (25).)

The relation of free energies to rates of oxidation seem also to be controlled by what Shaffer (265, 266) calls "the equi-valence-change principle," a theory of great importance for its biological implications. One example given by him will illustrate the theory: Ce^{+++} ions in N H_2SO_4 ($Ce^{+++} + 1\ e \rightleftharpoons Ce^{++}$, $E_0 = +1.431$ volt, Baur and Glasson (39)) oxidize Tl^+ ions ($Tl^{+++} + 2\ e \rightleftharpoons Tl^+$, $E_0 = +1.206$, Sherrill and Haas (267)) very slowly, although both are electroactive oxidation-reduction systems and the potential of the oxidant (Ce^{+++}) is high enough to oxidize the reductant (Tl^+). If to such a system $MnSO_4$ is added (manganese ions may exchange electrons one by one or two at a time: $Mn^{++++} + 1\ e\ Mn^{+++}$, $E_0 = +1.642$ volts; $Mn^{++++} + 2\ e \rightleftharpoons Mn^{++}$, $E_0 = +1.577$ volts, Grube and Huberich (142)), the oxidation of thallous ions by ceric ions will proceed rapidly. According to

Shaffer, the oxidation is slow because the oxidant, Ce^{+++}, is a one-electron system, while the reductant, Tl^{+}, is a two-electron system; such a reaction would require a trimolecular collision, an event of such rarity that, if in addition to a molecular encounter considerable activation is required, the velocity of reaction will be negligibly small. Manganese acts in this reaction as catalyst because it can lose and accept one or two electrons and hence can replace a trimolecular collision with a series of bimolecular collisions. For the same reason a system capable of semiquinone formation could also act as catalyst. Shaffer has indeed found that various dyes which oxidize in two steps (pyocyanine, other phenazines) may act as catalysts in the above mentioned oxidation. Shaffer's theory has found strong support in Michaelis' discovery of the two-step oxidation systems, systems which are found in cells and whose rôle as catalysts has been amply proved (pyridine-nucleotides, alloxazins, phenazines, quinones). Michaelis has embodied Shaffer's ideas in what he calls the "principle of compulsory univalent oxidation." According to him any oxidation can proceed only in successive univalent steps. The lack of reactivity toward oxidation in general would be due to the fact that usually the formation of the intermediate radical requires a step-up in free energy. The free radical may be taken as the equivalent to the activated state of the molecule. So far, experimental findings favor these conceptions. They resemble Brönsted's theory for acid-base catalysis (52) as enlarged by Pedersen (249); for, according to them, the reaction between an acid catalyst HA and a substrate S,

$$HA + S \rightarrow \text{Products} + HA,$$

takes place in the following steps:

$$HA + S \rightleftharpoons SH^{+} + A^{-}$$
$$SH^{+} + A^{-} \rightarrow \text{Products} + HA.$$

The Rôle of Oxidation-Reduction Systems in Cellular Respiration. The three groups of reversible oxidation-reduction systems which are found in cells act as components of the oxidizing enzyme systems. As a rule the sluggish systems act as oxidizing catalysts of the activated substrate; the electroactive systems as the electron mediators with molecular oxygen. For this reason it will be well to start by discussing the rôle of the sluggish systems, and end with that of electroactive systems.

Sluggish oxidation-reduction systems. The discovery by Warburg and his school that pyridine nucleotides are *actual* components of

oxidation enzymes has, indeed, been of first importance to the understanding of the mechanism of cellular respiration, for this group of substances can be taken as the best example of sluggish reversible systems.

The function of diphosphopyridine nucleotide (Euler's cozymase, Warburg's coenzyme II) in biological oxidations has been established in a number of contributions by Green and his co-workers, and Euler and his co-workers. A number of carbohydrate derivatives activated by their specific activating proteins have been shown to be oxidized, in isolated enzyme systems, with diphosphopyridine nucleotide as an electron mediator: alcohol, lactic and malic acids, formic acid, glutamic acid, triose phosphate, citric acid, glucose (Euler and associates, Green and Dewan (136), Dewan and Green (78), Meyerhof and Ohlmeyer (225), Anderson (6)). Diphosphopyridine nucleotide has an important function as mediator in the series of oxidation-reductions going on during carbohydrate fermentation. The electron transfer from the reduced nucleotide seems to be mediated mainly through a pyridine nucleotide oxidase, an enzyme discovered by Green and by Euler. The orientation and speed of these reactions will be governed by thermodynamic relationships, concentration of reactants, oxygen tension, electrolyte concentration, and other factors still unknown.

The rôle of triphosphopyridine nucleotide in cellular respiration is not yet known. It is indeed of extreme interest that this nucleotide which can mediate the oxidation of hexose-monophosphate when activated by the protein purified by Negelein and Haas (241), can also act as mediator for the further oxidation of phosphohexonic acid when in the presence of another protein (Warburg and Christian (309)). We are once more in the presence of a series of oxidations, the oxidation of glucose being performed by steps. Is the potential of triphosphopyridine nucleotide altered by union with the specific activating proteins, thus allowing a gradual oxidation of the split products of the original hexose-monophosphate? Further research is needed to answer this question.

Other oxidations where triphosphopyridine nucleotide acts as an electron mediator are the oxidation of glucose (Harrison (148)) and of glutamic acid (Euler, Adler and Eriksen (98)). Das (75) reports that both pyridine nucleotides may act as mediators of the oxidation of glucose.

It is remarkable that the respiratory function of the sluggish system, glutathione, discovered as long ago as 1921 by Hopkins, the chemical constitution of which was established in 1929, and its synthesis effected in 1935 (Harrington and Mead (147)), is still unsolved. Can gluta-

thione act as an electron mediator between an activated substrate and an electroactive catalyst? Its oxidation-reduction potential seems to be about that of diphosphopyridine-nucleotide; Hopkins and Elliott showed in 1931 (158) that the system could be reversibly oxidized and reduced by the liver of well-fed animals. Nevertheless, no experimental proof of the catalytic power of glutathione in biological oxidations has yet been offered. The same may be said of ascorbic acid. Both sluggish systems seem to act as mediators of the processes of synthesis.

Further work in the field of sluggish oxidation-reduction systems will doubtless bring about the discovery of other substances acting as mediators between the activated substrate and the electroactive catalyst.

A point of special importance, not yet studied, is whether the combination of pyridine nucleotides with the activating protein—a combination the reversible nature of which has been reported by the associates of Warburg—alters the oxidation-reduction potential of the system. If we assume that Clark's treatment of the effect of nitrogenous compounds on the potentials of hemin applies generally to systems combining reversibly, we may predict that the potentials of pyridine nucleotides will change on combination with proteins, i.e., the potential would become more positive if the reduced pyridine nucleotide associated more strongly with the protein, more negative if the oxidized component associated more strongly, or remain unaltered if the affinity for reductant and oxidant is the same. This study is of significance because it may determine whether the union of the activating protein to the pyridine nucleotide is an ordinary chemical combination or is only similar to those postulated in adsorption phenomena, leaving, in the latter case, the free energy of the system unaltered.

Enzymatic sluggish oxidation-reduction systems. It has been shown that although these systems are thermodynamically reversible, the rate of electron transfer does not become appreciable until an electroactive catalyst is added, which acts as an electron mediator. Given, therefore, a number of enzymatic oxidation-reduction systems of graded potentials and a number of electroactive systems of potentials lying between those of the sluggish systems, we may build a series of oxidation-reduction systems where the electron transfer may proceed from the system of more negative potential to the system of more positive potential. The enzymatic-sluggish oxidation-reduction systems may therefore act like the pyridine-nucleotides, i.e., as catalysts. Szent-Györgyi and his collaborators (284) have presented evidence of

the existence of such a series in their theory of C_4 dicarboxylic acids as catalysts for cellular respiration. According to them, triosephosphates are oxidized by oxalo-acetic acid; the malic acid formed in the oxidation is oxidized by fumaric acid; the succinic acid thus formed is oxidized by the cytochrome system. It is reasonable to believe that when enzymatic-sluggish reversible systems like those of Szent-Györgyi's system do exist cellular respiration as a whole may proceed through this series. Stare (273), Stare and Baumann (271) Boyland and Boyland (48), Greville (141), Leloir and Dixon (211), and others have offered evidence in favor of Szent-Györgyi's catalysts. (The papers from Szent-Györgyi's laboratory can be found discussed as a whole in Szent-Györgyi's book, *Studies on biological oxidation and some of its catalysts.*) The citric acid cycle postulated by Krebs and Johnson (191) would involve other reversible-sluggish systems, isocitric $\rightleftharpoons$ cisaconitic acid, a reversible reaction, according to Martius and Knoop (221), and the reaction, citric acid $\rightleftharpoons$ cisaconitic acid, also reversible, according to Breusch (51). Whether carbohydrate oxidation goes through Szent-Györgyi's cycle, Krebs' cycle, or through direct oxidation of pyruvic acid will depend not only on the presence or absence of the specific activating proteins and the electroactive electron mediators but also on all the factors which determine the orientation of these reversible reactions.

Electroactive oxidation-reduction systems. The property of electroactive systems of being autoxidizable, united to that of ready electron transfer with other oxidation-reduction systems, explains the function of this group. They may act as electron mediators between the activated substrate and molecular oxygen; between a sluggish mediator system (pyridine nucleotides, cytochrome *c*) and molecular oxygen; between enzymatic-sluggish oxidation-reduction systems (Borsook's coupled reactions or Szent-Györgyi's C_4 dicarboxylic acids); and finally, between one another (Theorell (290)), the system of more positive potential oxidizing that of more negative potential, in a series.

The catalytic power of an electroactive system in cellular respiration was demonstrated in 1928 (Harrop and Barron (149)). It was found at that time that the addition of methylene blue brought about a considerable increase in the respiration of mammalian erythrocytes, which was accompanied with an increased disappearance of glucose and diminution of lactic acid formation (Barron and Harrop (31)). The catalytic effect is insensitive to HCN (Harrop and Barron (149)) and to CO (Gerard (124)). It was found in all tissues which produced lactic acid in aerobic conditions: leucocytes, tumour tissues, echinoderm

eggs (Barron (24)), muscle (Gerard (123)), fish erythrocytes (Chapheau (59)), nerve (Gerard (122)), retina (Fleischmann and Kann (110)). Previous to these experiments *with living cells*, Meyerhof had discovered in 1917 (223) that addition of methylene blue to acetone-treated bacteria produced a rapid oxygen consumption; Fleisch (109) had shown in 1924 that the oxidation of succinic acid by an enzyme preparation from muscle, when inhibited by HCN, was restored on addition of methylene blue. This catalysis was explained as the result of direct electron transfer between the oxidizable substrate and dye, and between dye and atmospheric oxygen. Wendel (320) and Warburg, Kubowitz, and Christian (314), who discovered that the product of lactic acid oxidation was pyruvic acid, postulated a theory by which the dyes oxidize hemoglobin (in experiments with red cells) and that the methemoglobin thus formed acted as an oxidant of lactic acid. Using red cells and as catalysts dyes of a potential unable to oxidize hemoglobin, DeMeio et al. (76) showed that the catalytic effect of methylene blue could be taken as a model to explain the function of electroactive systems:

$$\text{Lactate}^- + \text{M.B.} \rightarrow \text{Pyruvate} + \text{L.M.B.} + 2\,\text{H}^+ \quad (1)$$
$$\text{L.M.B.} + \text{O}_2 \rightarrow \text{M.B.} + 2\,\text{O}^- \quad (2)$$
$$2\,\text{H}^+ + 2\,\text{O}^- \rightarrow \text{H}_2\text{O}_2 \quad (3)$$

In cells possessing catalase, H_2O_2 is immediately decomposed.

a. Iron-porphyrins. The systems containing iron-porphyrin as the active nucleus are by far the most important. In fact this nucleus is present in the cytochromes of Keilin, in Warburg's "Sauerstoffübertragende Ferment der Atmung," in most peroxidases (Kuhn, Hand, and Florkin (196), Keilin and Mann (175)), in catalase (Zeile and Hellström (336), Stern (276), Sumner and Dounce (281)). Furthermore the iron-porphyrin nucleus possesses the interesting property of forming three sorts of compounds each of them playing a fundamental part in cellular respiration. Iron-porphyrin may unite with a protein and thus combine reversibly with molecular oxygen without electron exchange (hemoglobin, myoglobin, possibly other heme-protein compounds not yet isolated). The loading and unloading of oxygen by these oxygen store pigments will depend on the value of the equilibrium constant (Barcroft (23), Millikan (234)); iron-porphyrin may combine with a protein forming a sluggish oxidation-reduction system, non-autoxidizable (cytochrome *c* and possibly other cytochromes); iron-porphyrin may combine with nitrogenous compounds giving autoxidiz-

able electroactive systems (hemochromogens, Keilin's "cytochrome oxidase"). Thus iron-porphyrin compounds may act as mediators between activated substrates and electroactive systems, and as final mediators with molecular oxygen.

The function of cytochromes has been recently discussed by Keilin (169) and Shibata (268). Cytochrome *c*, a sluggish reversible system, seems to act, like the pyridine-nucleotides, as electron mediator between the activated substrate and the autoxidizable catalyst; it acts in this way in the oxidation of succinic acid. When the three cytochromes are present in cells they may all take part in the oxidation-reduction series:

1. $2\,Fe^{++}$ cyt *b* + $2\,Fe^{+++}$ cyt *c* $\rightarrow$ $2\,Fe^{++}$ cyt *c* + $2\,Fe^{+++}$ cyt *b*; (E'_0 cyt *b*, -0.04 volt)
2. $2\,Fe^{++}$ cyt *c* + $2\,Fe^{+++}$ cyt *a* $\rightarrow$ $2\,Fe^{++}$ cyt *a* + $2\,Fe^{+++}$ cyt *c*; (E'_0 cyt *c*, $+\,0.255$ volt)
3. $2\,Fe^{++}$ cyt *a* + O_2 $\rightarrow$ Fe^{+++} cyt *a* + $2\,O^-$ (E'_0 cyt *a*, $+0.29$ volt)

Equation 3 is hypothetical. Whether cytochrome *a* is autoxidizable or not is not known with certainty, though Keilin and Hartree (174) have observed that part at least of cytochrome *a* is autoxidizable.

Whether Keilin's "cytochrome oxidase" or Warburg's "Sauerstoff-übertragende Ferment" is cytochrome *a* or a hemochromogen of higher potential is not known. The property of iron-porphyrins of combining reversibly with nitrogenous compounds to give oxidation-reduction systems of varied potential has made of them the almost universal mediators of electrons in the last steps of cellular respiration.

As hemochromogens combine reversibly with HCN and CO, the inhibition in oxidation produced by these two compounds was taken as an indication of oxidation with hemes as catalysts. However, Sato and Tamiya (262) have shown the existence of cells which although possessing some of these cytochromes had their respiration inhibited by HCN and not by CO, and of cells whose respiration was inhibited by neither. Finally, there are oxidations catalyzed by hemin, as the oxidation of unsaturated fatty acids, where HCN has no inhibiting effect (Barron and Lyman (36)).

Alloxazins. As soon as Warburg discovered his "Yellow Ferment" a series of papers appeared from different laboratories (see Wagner-Jauregg's review (300) and Euler's review (94)) maintaining that this system acted as electroactive mediator between molecular oxygen and

the next member in the series of oxidation-reduction systems (generally a pyridine nucleotide). It was even called "Methylenblau der Zelle." As an electroactive system, indeed, it may act as such under conditions where no other electroactive system of higher potential is present. But even in such cases, when there are cytochromes present, and the oxygen tension is lowered to that presumably existing in the cells, phosphoriboflavin protein is reoxidized through the cytochrome system (Theorell (290)). The function of alloxazins in cellular respiration remained unknown until 1938 when the two important contributions from Warburg's laboratory already referred to were published (one by Warburg and Christian (311) on d-amino acid oxidase; the other by Ball (18) on xanthine oxidase). The prosthetic group of these two oxidizing enzymes is made up of alloxazin-adenine-dinucleotides; when combined with the specific activating proteins these alloxazins oxidize the oxidizable substrate, the reduced alloxazin being reoxidized by atmospheric oxygen. Alloxazin dinucleotide from d-amino acid oxidase on combining with the protein of the yellow ferment actively oxidizes dihydrodiphosphopyridine nucleotide (reduced cozymase). The prosthetic group of Green's "coenzyme factor" (pyridine nucleotide oxidase) might be an alloxazin dinucleotide. Alloxazin mononucleotide seems to have no action as oxidation catalyst. Phosphoriboflavin protein acts as an electron mediator for reduced pyridine nucleotides (Haas (144)). Electron transfer from the reduced flavoprotein to fumaric acid (activated by the proper protein) has been shown by Laki (202); and Banga (21) has shown that it may act as electron mediator between the reactions, malic acid and fumaric acid. Thus alloxazins seem to act in highly organized cells as electron mediators between sluggish oxidation-reduction systems. (Its rôle in glycolysis is a consequence of this assumption.) As an electroactive system, it may act as the final mediator of electrons in the series, if 1, no electroactive systems of higher potential are present, or if 2, such systems have been rendered inactive. Examples of the first class are found in the respiration of a number of bacteria (Warburg and Christian (306), Fujita and Kodama (120)). Examples of the second class are provided by the residual respiration of mammalian tissues after addition of HCN (Dixon and Elliot (85)). It must be recalled that, in general, bacteria devoid of iron-porphyrin compounds live by fermentation, anaerobically, the flavoprotein acting as electron mediator; in the presence of oxygen, the flavin acts as catalyst for oxidations, an oxygen uptake ensues and

H_2O_2 starts piling up (because of the absence of catalase and absence of coupled oxidation where H_2O_2 may act as oxidant). As Warburg and Christian (307) remarked, this is an unphysiologic process.

Other electroactive systems. (See table 3.) After the discovery of the catalytic action of methylene blue on cellular respiration, a number of papers appeared in the literature (Friedheim (112–116) describing the catalytic effect of electroactive systems normally present in cells. As these experiments were performed with cells of very low respiration (generally mammalian red cells) it must be said that the rôle of these pigments in cellular respiration is still unknown. *B. pyocyaneus* contains cytochromes besides pyocyanine, and its respiration is inhibited by HCN (Yamagutchi (334)). They may act either as the last step towards oxygen or as mediators of coupled oxidations (Michaelis and Smythe (233)).

Kisch and his co-workers (182–184) have devoted a number of papers to the study of the catalytic effect of oxidation-reduction systems of quite positive potential (quinones, pyrocatechol, adrenaline). According to the authors they act as good catalysts for oxidative deamination. Hodel (155) reported on the effect of adrenaline on cellular respiration. This effect may be due to adenochrome formation which according to Green and Richter (139) is an oxidation product of adrenaline which acts itself as a reversible system. Blasschko, Richter, and Schlossman (41) speak of an HCN-insensitive system in tissues which oxidizes adrenaline to adrenochrome. Whether these quinones, of which there are a number of living systems, act as normal catalysts for biological oxidations is not known. Their rôle as catalysts in plant respiration has been suggested by Palladin (245) and Oparin (244). Amino acids are oxidized in tissue extracts (Bernheim and Bernheim (40), Krebs (188, 189), Keilin and Hartree (170)) by two groups of oxidizing enzymes: one, extractable, HCN-insensitive, oxidizes d-amino acids; the other, non-extractable, HCN-sensitive, oxidizes l-amino acids. The components of the second enzyme are not yet known.

Activating Proteins (Dehydrogenase; Dehydrase, Apodehydrase, Zwischenferment). Gonococci suspended in 0.1 M NaF have the ability to oxidize, in the presence of oxygen, lactic acid to pyruvic acid (Barron and Hastings (32)). No other oxidizing enzyme is present in this suspension. On heating the suspension for 2 hours at 52°, the suspension was unable to oxidize lactate; the oxidation started as soon as an electroactive oxidation-reduction system of suitable potential was added. On heating to 70°, the oxidation did not proceed even on addition of the

electroactive system. The oxidation of lactic acid to pyruvic acid required thus the action of two factors equally important: one concerned with the activation of the lactate molecule; the other concerned with the oxidation of the activated lactate. The activation of the lactate molecule occurs at the surface of the activating enzymes, a protein, as only the lactate adsorbed by the enzyme can be oxidized.

Wieland (see his book (321)) and Thunberg (see his latest review (295)) deserve full credit for having been the pioneers for the discovery of the activating proteins, the isolation of some of which has been brilliantly performed in Warburg's laboratory; Negelein and Gerischer (240) prepared from yeast a protein (Zwischenferment) which specifically activated hexosemonophosphate; Negelein and Wulff (242) isolated from yeast a protein which specifically activated the reversible reaction alcohol $\rightleftharpoons$ acetaldehyde. (This is a clear example of the need for dropping names suceptible of misconception: this protein is called by Euler and his school "alcohol dehydrogenase," while Negelein calls it acetaldehyde "reductase." The protein may act either as "dehydrogenase" or "reductase" on altering the conditions which determine the value of the equilibrium constant.) Warburg and Christian (309) isolated a protein which activates the further oxidation of phosphohexonic acid. The activating protein of d-amino acid oxidase was isolated by Warburg and Christian (311). Ball (18) is working on the isolation of the activating protein of xanthine oxidase.

The specificity of the activating proteins has been satisfactorily demonstrated. These proteins may possess a species specificity, or a group specificity. Warburg's protein for the oxidation of hexose monophosphate seems to be the clearest example of the first group. Bernheim's extensive studies on the enzymes concerned with the oxidation of amino acids provide examples of group specificity. The enzymes obtained from milk, one able to oxidize purines ("xanthine oxidase"), the other able to oxidize aldehydes ("aldehyde oxidase") show less specificity. Considered by Wieland and Macrae (322) as two different enzymes, they have been shown by Booth (42) to be one enzyme. Here we have an enzyme activating purines (hypoxanthine, xanthine, and six other purines) and aldehydes.

Whether the protein is only a "colloidal carrier" of the pyridine-nucleotides and systems of similar oxidation-reduction properties, as Warburg and his school maintain, or whether it acts by activating the oxidizable molecule, i.e., altering the electronic configuration of the substrate in such a manner that it becomes ready to exchange electrons

with the pyridine nucleotides and systems analogous to them, is not yet known. Further work in this direction is necessary. Of special interest is the discovery of Hopkins and Morgan (159) that the activating protein for the oxidation of succinic acid loses its activity on treatment with oxidized glutathione, the activity being restored on further treatment with reduced glutathione. Similar findings have been reported by Rapkine (255) with triosephosphate oxidase. It would seem that an SH group in the protein is essential for its activity. (See Hellerman's review (150) on "Reversible inactivations of hydrolytic enzymes," where he deals with similar phenomena.) Wagner-Jauregg and Möller (299) have also reported that glutathione activates the activating protein of alcohol oxidase. Does the hypothetical system, 2 Protein SH $\rightleftharpoons$ Protein S-S-Protein + $2H^+$, act in the same way as the pyridine nucleotides?

Metallo-protein Complexes. When the oxidation-reduction potential of the oxidizable substrate approaches that of the oxygen potential the oxidation enzymes become less complicated. The oxidizing catalyst, generally a heavy metal, combines more firmly with the protein giving metallo-protein complexes. *Peroxidase* seems to be an iron-porphyrin-protein complex (Kuhn, Hand, and Florkin (196), Keilin and Mann (175)). *Catalase* has also been considered an iron-porphyrin-protein complex (Stern (277), Sumner and Dounce (281), Keilin and Hartree (171)). However, Agner reports (4) that catalase preparations of higher purity than those of Sumner's "crystalline catalase" contain copper (0.02–0.03 per cent) besides iron (0.085 per cent). Whether catalase contains two metallo-protein complexes whereby the ferrous complex formed on the decomposition of H_2O_2 is oxidized by the cupric complex, and the cuprous complex thus formed oxidized by molecular oxygen is not yet known. *Polyphenol oxidase* is a copper-protein complex containing 0.165 per cent copper (Kubowitz (194), Keilin and Mann (173)). *Uricase*, the enzyme which oxidizes uric acid to allantoin, seems to be an iron-protein complex (Davidson (75a)). In all these enzymes oxidation is performed by electron transfer between the metallo-complex (the -ic form) and the oxidizable substance and between the metallo-complex (the -ous form) and molecular oxygen. Further research in this group of enzymes may discover the existence of other metallo-protein complexes acting as catalysts in the oxidation of those substances of highly positive potentials.

The Oxidation-Reduction Series in Cellular Respiration. It has been shown that the cell possesses three kinds of reversible oxidation-reduc-

tion systems: the electromotively active systems, the sluggish systems, and the enzymatic-sluggish systems. As a rule, only the electromotively active systems transfer electrons to oxygen; these systems also transfer electrons between the sluggish systems. All of them, however, by virtue of their reversibility *may* take part in cellular respiration by transferring electrons from foodstuffs towards oxygen, their rôle being that of electron mediators or catalysts. Thus an oxidation-reduction series is established. Free energy relationships of necessity, will decide whether system *A may be* oxidized by system *B*. They may also determine the rate of reaction if we accept the postulate that the specific rate constant, k_1, of these reactions depends on the free energies of the activated systems (Gershinowitz (126)). The relation of free energy to rate of oxidation may also be conditioned according to Shaffer's equivalence principle and Michaelis' principle of compulsory univalent oxidation.

Activated foodstuffs seem to be oxidized in the cell in such a way that the release of energy of oxidation is performed through a series of reversible oxidation-reduction systems. Energy is thus released by gradual steps, the number of steps being determined by the number of systems making up the series.

The concept of the oxidation-reduction series explains the great number of coupled oxidations going on not only during respiration but also during fermentation (Quastel and Wooldridge (254), Borsook (43), Green, Stickland, and Tarr (140), Dewan and Green (78), Needham and Pillai (239), Meyerhof (224)). From the extensive literature, two examples may be taken to illustrate the mechanism of such a series. Borsook was the first to demonstrate that formate and pyruvate, in the presence of their respective activating enzymes, do not react; the addition of an electroactive catalyst of a potential value between that of the other two systems produced the oxidation of formate and reduction of pyruvate. There is in this case the following oxidation-reduction series:

$\text{Pyruvate}^- + 2\,H^+ + 2\,e \rightarrow \text{Lactate} -$	E'_0	(pH 7.0)	$-$ 0.180 v.
Methylene violet $+ 2\,H^+ + 2\,e \rightarrow$ Leuco methylene violet	E'_0	"	$-$ 0.260
$\text{Formate}^- + H_2O \rightarrow HCO_3 + 2\,H^+ + 2\,e$	E'_0	"	$-$ 0.410

In this series, the electroactive system, reduced on the oxidation of formate, is oxidized on the reduction of pyruvate.

Another interesting example is given by Szent-Györgyi (283). In the

presence of their respective activating enzymes and of the reversible systems phosphoriboflavin-protein, cytochrome c and "cytochrome oxidase," malate is oxidized to oxaloacetate, fumarate reduced to succinate, and succinate reoxidized to fumarate. There is here the following oxidation-reduction series (on the assumption that cytochrome a equals cytochrome oxidase):

Ferricytochrome $a \rightarrow$ Ferrocytochrome $a + 1\ e$	$E'_0 = +0.29$ volt
Ferricytochrome $c \rightarrow$ Ferrocytochrome $c + 1\ e$	$E'_0 = +0.255$ volt
Fumarate$^=$ $+ 2\ H^+ + 2\ e \rightarrow$ Succinate$^=$	$E'_0 = 0$
Phosphoriboflavin protein $+ 2\ H^+ + 2\ e \rightarrow$ Red flavoprotein	$E'_0 = -0.063$ volt
Malate$^=$ $\rightarrow$ Oxaloacetate$^=$ $+ 2\ H^+ + 2\ e$	$E'_0 = -0.169$ volt

Interspersed in this series are two electroactive systems: (1) phosphoriboflavin-protein and (2) cytochrome a (the "cytochrome oxidase"), the energy of oxidation being transferred stepwise.

The Mechanism of Enzymatic Oxidations. Foodstuff under the conditions existing in living cells is not oxidized by mild oxidizing agents. On collision with a specific protein molecule, the activating protein (dehydrogenase, apodehydrase, *Zwischenferment*), it becomes *activated,* i.e., a change in its electronic structure occurs, by which the electrons become ready to be transferred through the oxidation-reduction series. In the simplest type of oxidation enzyme the activated substrate may be oxidized directly by molecular oxygen. In such a case the series would be made of only two elements, the release of energy being made wastefully, suddenly. Keilin and Hartree (170) believed that uricase amino acid oxidase, and xanthine oxidase are examples of this group. Although such a type may exist, effort must be made to find the systems which mediate the transfer of electrons between the oxidizable substrate and molecular oxygen. In fact, Warburg and Christian (319) and Ball (18) have recently isolated d-amino acid oxidase and xanthine oxidase, which contain two systems: the activating protein and alloxazin-adenine-dinucleotide. Davidson (75a) has found that uricase contains 0.15 to 0.2 per cent iron. This group may then be considered doomed to disappear.

In the second type, the oxidizing enzyme is made up of two components, the activating protein and the electroactive system: d-amino acid oxidase and xanthine oxidase are examples of this group. The activated substrate is oxidized by alloxazin-adenin-dinucleotide; reduced alloxazin is oxidized by molecular oxygen.

The third type requires the presence of an electromotively active

system which will transfer electrons from the oxidizable substrate through the cytochrome system to oxygen. The length of the series will be determined by the number of hemochromogens existing in the system. Succino-oxidase may be given as an example. In the course of the phylogenetic evolution of the cell an oxidizing enzyme may add more components. Thus α-hydroxyoxidase in yeast and gonococci require only the activating protein and electroactive catalyst (Green and Brostreaux (135), Barron and Hastings (32)), but in animal tissues the enzyme adds a sluggish reversible system, diphosphopyridine nucleotide, as another component (Banga and Szent-György (22) Green and Brostreaux (135)).

A fourth type of enzyme requires the presence of two reversible oxidation-reduction systems: one, of the sluggish type, non-autoxidizable by atmospheric oxygen; the other, of the electroactive type, autoxidizable by atmospheric oxygen. The well-known Warburg system which oxidizes hexosemonophosphate may be given as an example. Activated hexose monophosphate is oxidized by triphospyridine nucleotide; reduced triphosphopyridine is oxidized by phosphoriboflavin-protein; reduced flavoprotein is finally oxidized by oxygen. The existence of this type of oxidation seems limited to cells with no iron-porphyrins.

A fifth type of oxidation requires the presence of four reversible systems: two sluggish, a pyridine nucleotide and cytochrome *c*; two electroactive, alloxazin and a hemochromogen (Warburg's Atmungsferment or Keilin's cytochrome oxidase). Theorell has shown that at the low oxygen pressure of tissues the direct oxidation of phosphoriboflavin-protein by oxygen is not very rapid, but that reduced flavoprotein can reduce cytochrome through which the reaction proceeds more rapidly. To this type may be grouped all those systems studied by Green and Euler where the enzyme components are: protein, pyridine nucleotide, the cytochrome system.

Catalase, peroxidase, and polyphenol oxidase seem to require no activating protein. The prosthetic group, iron-porphyrin or copper form complex compounds with the protein, the complex being reduced by the oxidizable substrate, and the reduced complex being oxidized by molecular oxygen (VI type).

As the living cell becomes more complicated and leaves the path of fermentation for that of oxidation, it develops a series of reversible systems of graded energies, systems which deliver smoothly the energy provided by the final reduction of oxygen. The series of oxidation-reduction systems of the cell becomes longer and longer.

Figure 1 gives the oxidation-reduction series as found in heart muscle,

made up of electroactive systems, sluggish systems, and enzymatic-sluggish systems.

Decarboxylative Oxidations. The end products of cellular respiration are CO_2 and H_2O. Oxygen is taken up and CO_2 is given out. In which

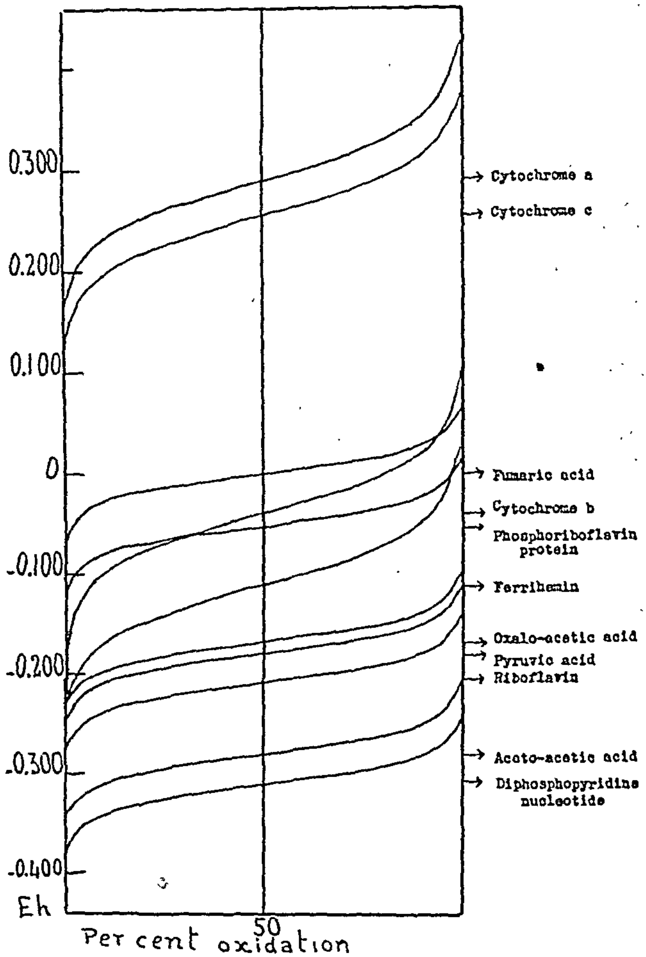


Fig. 1. Reversible oxidation-reduction systems present in mammalian heart. E_h in volts; pH 7.0

steps of the oxidation-reduction series this CO_2 is given out we barely know. Some of this CO_2 may come through intermolecular oxidation-reductions of ketonic acids (Krebs and Johnson (191), Elliot et al. (91, 92)); some by interaction between α-ketonic acids and amino acids,

as Braunstein and Kritzmann (50) have shown; some by the oxidation of saturated fatty acids; some by the direct oxidation of pyruvic acid, the *main intermediate product of carbohydrate metabolism.* The decarboxylative oxidation of pyruvic acid was demonstrated with the aid of bacteria (gonococci) which oxidized it to only acetic acid and CO_2 (Barron and Miller (38)). This process required an activating protein and an iron compound which probably acts as a chain-reaction catalyst.

Peters and his co-workers (250) have contributed greatly to our knowledge of the mechanism of this oxidation in their studies of the oxidation of pyruvic acid by avitaminotic pigeon's brain. The oxidation of pyruvic acid was increased on addition of thiamin (vitamin B_1). Lohman and Schuster's discovery that "cocarboxylase," the "coenzyme" for pyruvic acid decarboxylation in yeast, was a diphosphothiamin, was followed by Lipman's (214) demonstration that this diphosphothiamin takes part in the enzyme complex concerned with the oxidation of pyruvic acid. In experiments with bacteria it has been shown (Barron and Lyman (37)) that thiamin must be phosphorylated before becoming part of the pyruvic acid oxidizing system. It takes part also in the dismutation of pyruvic acid. Is diphosphothiamin, like the pyridine nucleotides, reduced by pyruvic acid and reoxidized by an electroactive catalyst? This possibility, pointed out by Clark (64), has not yet been demonstrated.

Work on other decarboxylative oxidations is sorely needed.

The Oxidation and Synthesis of Amino Acids. The oxidation of amino acids starts with the intermediate formation of imino acids and the final formation of keto acids and NH_3:

$$\begin{array}{c} R \\ | \\ HC{-}NH_2 \\ | \\ COOH \end{array} \xrightarrow{+O_2} \begin{array}{c} R \\ | \\ C{=}NH \\ | \\ COOH \end{array} \xrightarrow{+H_2O} \begin{array}{c} R \\ | \\ C{=}O \\ | \\ COOH \end{array} + NH_3$$

The first process is a reversible oxidation-reduction system:

$$\begin{array}{c} R \\ | \\ HC{=}NH_2 \\ | \\ COOH \end{array} \rightleftharpoons \begin{array}{c} R \\ | \\ C{=}NH \\ | \\ COOH \end{array} + 2H^+ + 2e$$

The second process, a hydrolysis, proceeds spontaneously. In the oxidation of d-amino acids (by the enzyme purified by Warburg and

Christian), d-alanine, for example, is oxidized by alloxazin dinucleotide and reduced alloxazin is oxidized by atmospheric oxygen:

(1) CH_3CHNH_2COOH + Alloxazin $\rightleftharpoons$ Reduced alloxazin + $CH_3C{=}OCOOH$ + NH_3

(2) Reduced alloxazin + $O_2 \rightleftharpoons$ Alloxazin + H_2O_2

Of these two reversible systems, the system alanine $\rightleftharpoons$ ammonium pyruvate is an enzymatic sluggish system (Wurmser and Filitti-Wurmser (329)), and the alloxazin system is an electroactive system. By virtue of their reversibility there will be either oxidation or synthesis of amino acid, depending on the values of the equilibrium constants.

In the oxidation of 1(+) glutamic acid (Euler *et al.* (100a)) the oxidative enzyme is made up of an activating protein (dehydrogenase), phosphopyridine nucleotides (triphosphopyridine nucleotide in yeast and *B. coli*, di- and triphosphopyridine nucleotides in animal tissues), and probably alloxazin dinucleotide. There are present in this series the three groups of reversible systems: (1) enzymatic sluggish (glutamic acid $\rightleftharpoons$ NH_3Ketoglutarate + $2H^+$ + 2 e); (2) sluggish (diphosphopyridine nucleotide); (3) electroactive (alloxazin). The potential of system (1) at pH 7.0 (30°C.) is about −0.181 volt (calculated from equilibrium data given by Euler *et al.*). The potential of system (2) at the same pH is about −0.314 volt. The potential of system (3) is not known. These values explain Euler's findings, namely, that diphosphopyridine nucleotide reduced by the oxidation of either alcohol or glucose (activated by their respective activating proteins) will readily reduce ammonium ketoglutarate to glutamic acid. It is Euler's opinion that in the liver (where alcohol and glucose oxidases are abundant) the reaction is oriented towards the synthesis of amino acids. Braunstein and Kritzman (50) found that muscle tissue forms and breaks down amino acids by intermolecular transfer of NH_2 groups by means of an enzyme. In the presence of glutamic acid and pyruvic acid, glutamic acid was oxidized to α-ketoglutaric acid, while pyruvic acid was converted to alanine. There are here (aside from diphosphopyridine nucleotide) two enzymatic sluggish systems reacting undoubtedly through some electroactive system present in muscle. At pH 7.0, the E'_0 values of these systems are:

(1) Alanine $\rightleftharpoons$ Ammonium pyruvate, −0.040 volt

(2) Glutamic acid $\rightleftharpoons$ Ammonium ketoglutarate, −0.181 volt.

From the values of the potentials it may be predicted that glutamic acid will be oxidized and pyruvic acid will be converted to alanine, another method of amino acid synthesis.

In the third kind of amino acid synthesis, in which there is formation of an acetyl derivative (Knoop (185a), du Vigneaud and Irish (298a)), essentially the same-oxidation-reduction processes are involved: amino acid is synthesized by a series of oxidation-reductions involving pyruvic acid and NH_3. Pyruvic acid, a breakdown product of carbohydrate metabolism, takes part in the synthesis of amino acids and proteins by its reductive amination, a process which is easily performed because of the positive value of the potential of the reversible system alanine $\rightleftharpoons$ ammonium pyruvate.

Orientation of Reactions. The energy required for the maintenance of cell activities comes from foodstuff. The cell either splits it or burns it. We call the first process fermentation, the second oxidation. The cells belonging to the lowest group in the phylogenetic scale are undoubtedly those capable of "vie sans air," as Pasteur (247) called it, in which the energy requirements are met by fermentation processes. Fermentation is much the simpler of the two processes, but at the same time the less economical because the largest part of the energy of the carbohydrate molecule remains in the lactic acid or the alcohol molecule. The greater energy requirements of morphologically and physiologically complex organisms introduced atmospheric oxygen into the metabolism of living matter and thus respiration started. As soon as respiration appeared we had the problem of orientation of reactions. Aerobic cells in the absence of air split carbohydrate to lactic acid or alcohol; in the presence of air, oxygen is consumed, CO_2 produced, and fermentation stopped. The intimate relation of these two processes, a relation whose quantitative interdependence was discovered by Pasteur (now known as the Pasteur reaction) may be considered the integral of these orientations of reactions. Moreover, the only difference between fermentation and respiration is that the former is a series of phosphorylated oxidation-reductions with no oxygen, while the latter is a series of oxidation-reductions some of them decarboxylative, with oxygen as the final oxidation agent. Where does the linkage between fermentation and respiration begin? The numerous inhibition experiments leaving respiration intact and increasing aerobic fermentation, the so-called inhibition of the Pasteur reaction (Krah (186), Warburg (301), Dickens (79)) have brought no light. Bumm, Appel, and Fehrenbach's (53) claim that reduced glutathione inhibits the Pasteur reaction specifically and reversibly has been denied by Baker (12). Lipmann's (213) conclusions from experiments on the inhibition of fermentation by reversible dyes have found another explanation in

experiments of the same kind by Michaelis and Smythe (233). It has been generally agreed that respiration and fermentation are always united and that carbohydrates cannot be directly oxidized without preliminary anaerobic scission. The discovery of enzymes able to oxidize glucose directly (Boysen-Jensen (49), Müller (236), Harrison (148)) and the fact that iodoacetic acid may inhibit glycolysis without influencing respiration (Lundsgaard (218), Shorr, Barker, and Malani (269)) have been serious objections to the validity of this theory. Though the possibility of direct oxidation of glucose in some cases is admitted, it seems that in most cases glucose is first split before undergoing oxidation.

TABLE 5

The metabolism of carbohydrates

Products formed during glycolysis:

Hexose $\xrightarrow[\text{glucose oxidase}]{\text{oxidation}}$

Hexose monophosphate $\xrightarrow[\text{hexose monophosphate oxidase}]{\text{oxidation}}$

Triose $\xrightarrow[\text{triose oxidase}]{\text{oxidation}}$ further oxidation through $\nearrow$ C_4 dicarboxylic acid catalysis; $\searrow$ citric acid catalysis

Pyruvic acid $\xrightarrow[\alpha\text{-ketonoxidase}]{\text{oxidation}}$

Lactic acid $\xrightarrow[\alpha\text{-hydroxyoxidase}]{\text{oxidation}}$

The link between fermentation and respiration (the reader interested in a review of the Pasteur reaction may see two recent articles by Dixon (83) and by Burk (54)) may be produced at the different steps of the series of reactions of carbohydrate metabolism which in fermentation end either in lactic acid or alcohol. One of these steps may be the oxidation of hexose phosphates; another may be the oxidation of trioses through coupled oxidations with the reversible C_4 dicarboxylic acids (Szent-Györgyi (283)) or through the reversible reactions in citric acid oxidation (Krebs and Johnson (192)). The link may occur also after the formation of pyruvic acid, which in the presence of oxygen may be directly oxidized into acetic acid and CO_2 (table 5).

It is at once apparent that the orientation of reactions will depend on

a variety of factors. The number of oxidation-reduction systems making up the series (enzymatic-sluggish systems, pyridine-nucleotides, alloxazins, iron-porphyrin compounds) will determine the degree of complexity of the Pasteur reaction; the orientation will be affected by the oxygen tension (Kempner (179) Leiner (210)), temperature (Kubowitz (193), Nakashima (238), Dixon (82)), concentration of electrolytes (Ashford and Dixon (10), Chang, Gerard, and Shaffer (58)), and hormones (Cohen and Gerard (69)).

To demonstrate the complexities which surround the orientation of reactions in living cells, the metabolism of pyruvic acid may be taken as an example, pyruvic acid being selected because it occupies a central position in oxidation as well as in fermentation. It is the primary oxidation product of triose and the mother substance of lactic acid as well as of alcohol. Activated by the activating proteins of oxidation enzymes, it becomes extremely reactive. If a cell performs its respiration through a small oxidation-reduction series, for example, *Streptococcus hemolyticus*, pyruvic acid is oxidized to acetic acid and CO_2 in the presence of oxygen (Barron and Jacobs (35)), while in its absence it suffers an intramolecular oxidation-reduction splitting into acetic acid and formic acid. In another cell, gonococcus, where there is an enzyme for the reversible oxidation-reduction system ($\text{Pyruvate}^- + 2\,e + 2\,H^+ \rightleftharpoons \text{Lactate}^-$), in the presence of oxygen pyruvic acid is oxidized as before into acetic acid and CO_2; in the absence of air it is reduced partly into lactic acid and oxidized partly into acetic acid and CO_2 (Krebs (190)). It is obvious that pyruvic acid in these cells will orient its metabolism according to the oxygen tension, pH, concentration of reactants, and other factors which may influence the direction of these reactions. In more highly differentiated cells (muscle or liver), pyruvic acid may combine with amino acids reversibly (the amino group and 2 H of glutamic acid are transferred to pyruvic acid with formation of alanine (Braunstein and Kritzmann (50)); with other ketonic acids (Krebs and Johnson (190), Weill-Malherbe (319)). As the number of oxidation enzymes in the cell increases, the reactivity of pyruvic acid apparently increases for there will be an orientation of reactions of the first split products of pyruvic acid, giving puzzles for the imagination of those working in intermediary metabolism. (See the interesting papers of Elliott et al. (91, 92).)

If pyruvic acid, a split product of carbohydrate metabolism, can orient its cell reactions in so many different ways, it is easy to understand why the mechanism of the Pasteur reaction is still unknown.

(45) Borsook, H., E. L. Ellis and H. M. Huffman. J. Biol. Chem. **117**: 281, 1937.
(46) Borsook, H. and G. Keighley. Proc. Nat. Acad. Sc. **19**: 875, 1933.
(47) Borsook, H. and H. F. Schott. J. Biol. Chem. **92**: 535, 1931.
(48) Boyland, E. and M. E. Boyland. Biochem. J. **30**: 877, 1936.
(49) Boysen-Jensen, P. Biochem. Ztschr. **236**: 211, 1931.
(50) Braunstein, A. E. and M. G. Kritzmann. Biokhimiya **2**: 242, 1937.
(51) Breusch, F. L. Ztschr. physiol. Chem. **250**: 262, 1937.
(52) Brönsted, J. N. Chem. Rev. **5**: 231, 1928.
(53) Bumm, E., H. Appel and K. Fehrenbach. Ztschr. physiol. Chem. **223**: 207, 1934.
(54) Burk, D. Some fundamental aspects of the cancer problem. Science Press, 1937.
(55) Bushmakin, I. N. and K. I. Kuchinskaya. Trudy Gosud. Opyt. Zavoda Sint. Kauchuka, Litera B. Synthetic Rubber **4**: 160, 1935.
(56) Cannan, R. K. Biochem. J. **20**: 927, 1926.
(57) Cannan, R. K. Biochem. J. **21**: 184, 1927.
(58) Chang, T. H., R. W. Gerard and M. Shaffer. Am. J. Physiol. **101**: 19, 1932.
(59) Chapheau, M. Bull. Stat. Biol. Arcachon **27**: 29, 1930.
(60) Chow, B. F. J. Am. Chem. Soc. **57**: 1437, 1935.
(61) Chow, B. F. and S. E. Kamerling. J. Biol. Chem. **104**: 69, 1934.
(62) Clark, W. M. Pub. Health Repts. **38**: 443, 1923.
(63) Clark, W. M. Medicine **13**: 207, 1934.
(64) Clark, W. M. J. Applied Physics **9**: 97, 1938.
(65) Clark, W. M. and B. Cohen. Pub. Health Repts. **38**: 666, 1923.
(66) Clark, W. M., B. Cohen and H. D. Gibbs. Pub. Health Repts. **40**: 1131, 1925.
(67) Clark, W. M., J. F. Taylor, T. H. Davies and R. Lewis. Compt. Rend. Lab. Carlsberg Sér. Chim. **22**: 129, 1938.
(68) Cohen, E. and C. A. Elvehjem. J. Biol. Chem. **107**: 97, 1934.
(69) Cohen, R. A. and R. W. Gerard. J. Cell. and Comp. Physiol. **10**: 223, 1937.
(70) Conant, J. B. Chem. Rev. **3**: 1, 1926.
(71) Conant, J. B., G. A. Alles and C. O. Tongberg. J. Biol. Chem. **79**: 89, 1928.
(72) Conant, J. B. and C. O. Tongberg. J. Biol. Chem. **86**: 733, 1930.
(73) Coolidge, T. B. J. Biol. Chem. **98**: 755, 1932.
(74) Corran, H. S. and D. E. Green. Nature **142**: 149, 1938.
(75) Das, N. B. Ztschr. physiol. Chem. **238**: 269, 1936.
(75a) Davidson, J. N. Biochem. J. **32**: 1386, 1938.
(76) De Meio, R. H., M. Kissin and E. S. G. Barron. J. Biol. Chem. **107**: 579, 1934.
(77) Dewan, J. G. and D. E. Green. Nature **140**: 1097, 1937.
(78) Dewan, J. G. and D. E. Green. Biochem. J. **31**: 1074, 1937.
(79) Dickens, F. Biochem. J. **28**: 537, 1934; **30**: 1323, 1936.
(80) Dilling, W. J. Atlas der Kristallformen und der Absorptionsbänder der Hämochromogene. Stuttgart, 1910.

a variety of factors. The number of oxidation-reduction systems making up the series (enzymatic-sluggish systems, pyridine-nucleotides, alloxazins, iron-porphyrin compounds) will determine the degree of complexity of the Pasteur reaction; the orientation will be affected by the oxygen tension (Kempner (179) Leiner (210)), temperature (Kubowitz (193), Nakashima (238), Dixon (82)), concentration of electrolytes (Ashford and Dixon (10), Chang, Gerard, and Shaffer (58)), and hormones (Cohen and Gerard (69)).

To demonstrate the complexities which surround the orientation of reactions in living cells, the metabolism of pyruvic acid may be taken as an example, pyruvic acid being selected because it occupies a central position in oxidation as well as in fermentation. It is the primary oxidation product of triose and the mother substance of lactic acid as well as of alcohol. Activated by the activating proteins of oxidation enzymes, it becomes extremely reactive. If a cell performs its respiration through a small oxidation-reduction series, for example, *Streptococcus hemolyticus*, pyruvic acid is oxidized to acetic acid and CO_2 in the presence of oxygen (Barron and Jacobs (35)), while in its absence it suffers an intramolecular oxidation-reduction splitting into acetic acid and formic acid. In another cell, gonococcus, where there is an enzyme for the reversible oxidation-reduction system ($\text{Pyruvate}^- + 2\,e + 2\,H^+ \rightleftharpoons \text{Lactate}^-$), in the presence of oxygen pyruvic acid is oxidized as before into acetic acid and CO_2; in the absence of air it is reduced partly into lactic acid and oxidized partly into acetic acid and CO_2 (Krebs (190)). It is obvious that pyruvic acid in these cells will orient its metabolism according to the oxygen tension, pH, concentration of reactants, and other factors which may influence the direction of these reactions. In more highly differentiated cells (muscle or liver), pyruvic acid may combine with amino acids reversibly (the amino group and 2 H of glutamic acid are transferred to pyruvic acid with formation of alanine (Braunstein and Kritzmann (50)); with other ketonic acids (Krebs and Johnson (190), Weill-Malherbe (319)). As the number of oxidation enzymes in the cell increases, the reactivity of pyruvic acid apparently increases for there will be an orientation of reactions of the first split products of pyruvic acid, giving puzzles for the imagination of those working in intermediary metabolism. (See the interesting papers of Elliott et al. (91, 92).)

If pyruvic acid, a split product of carbohydrate metabolism, can orient its cell reactions in so many different ways, it is easy to understand why the mechanism of the Pasteur reaction is still unknown.

CONCLUSION

In this review, cellular respiration has been considered in the light of thermodynamic and kinetic studies as well as the extensive research on the isolation of the oxidation enzymes. From these studies it seems reasonable that oxidizing enzymes in general are made up of two components, a protein and one or more reversible oxidation-reduction systems, the protein combining with varying degrees of affinity with the reversible system. When a group of these enzymes and their oxidizable substrates act together (oxidation-reductions in glycolysis, coupled oxidations, Szent-Györgyi's C_4 carboxylic acid catalysts, Krebs' citric acid catalysis, etc.) the oxidation reactions proceed, as a rule, in a series, the system of more negative potential being oxidized by the reduction of the system of more positive potential, and so on, in a series of graded steps up to the reduction of molecular oxygen. So, like locks in a canal, these reversible systems release gradually the oxidation energy.

The reversibility of many of these oxidation-reduction reactions (systems found among the oxidation products of carbohydrates, amino acids, and fatty acids) is one of the most important factors determining the orientation of reactions. According to the conditions which regulate the values of the equilibrium constants the reactions may be oriented toward either the oxidation or the synthesis of carbohydrates, proteins, and fats.

There is still, however, considerable work to be done in this field. Our knowledge of the enzymes which produce the oxidation of fats, for example, is nil. Little, too, is known of the mechanisms of those agents, such as hormones and vitamins, which control the speed and the direction of biological oxidations.

When applying these studies with isolated enzymes to living cells, we must guard against unfortunate generalizations, which so often are made as soon as a reversible oxidation-reduction system is discovered. We must proceed with the utmost caution, having always in mind the variables which influence the orientation and speed not only of a given reaction but of all those reactions which in the living cell end in the consumption of oxygen and the production of CO_2.

REFERENCES

(1) ADLER, E., H. v. EULER AND H. HELLSTRÖM. Sr. Vet. Akad. Ark. Kemi **12**: 1, 1937.

(2) ADLER, E., H. HELLSTRÖM AMD H. v. EULER. Ztschr. physiol. Chem. **242**: 225, 1936.

(3) ADLER, E. AND M. SREENIVASAYA. Ztschr. physiol. Chem. **249**: 24, 1937.
(4) AGNER, K. Biochem. J. **32**: 1702, 1938.
(5) ALTSCHUL, A. M. AND T. H. HOGNESS. J. Biol. Chem. **124**: 25, 1938.
(6) ANDERSON, B. Ztschr. physiol. Chem. **225**: 57, 1934.
(7) ANSON, M. L. AND A. E. MIRSKY. J. Physiol. **60**: 50, 1925.
(8) ANSON, M. L. AND A. E. MIRSKY. J. Gen. Physiol. **14**: 43, 1930.
(9) ANSON, M. L. AND A. E. MIRSKY. Physiol. Rev. **10**: 506, 1930.
(10) ASHFORD, C. A. AND K. C. DIXON. Biochem. J. **29**: 157, 1935.
(11) AUSTIN, J. H. AND D. L. DRABKIN. J. Biol. Chem. **112**: 67, 1935.
(12) BAKER, Z. Biochem. J. **31**: 960, 1937.
(13) BALL, E. G. J. Biol. Chem. **106**: 515, 1934.
(14) BALL, E. G. J. Biol. Chem. **114**: 649, 1936.
(15) BALL, E. G. J. Biol. Chem. **118**: 219, 1937.
(16) BALL, E. G. J. Am. Chem. Soc. **59**: 2071, 1937.
(17) BALL, E. G. Biochem. Ztschr. **295**: 262, 1938.
(18) BALL, E. G. Science **88**: 131, 1938.
(19) BALL, E. G., T. T. CHEN AND W. M. CLARK. J. Biol. Chem. **102**: 691, 1933.
(20) BANCROFT, W. D. AND A. B. GEORGE. J. Physiol. Chem. **35**: 2194, 1931.
(21) BANGA, I. Ztschr. physiol. Chem. **249**: 205, 1937.
(22) BANGA, I. AND A. SZENT-GYÖRGYI. Ztschr. physiol. Chem. **217**: 39, 1933.
(23) BARCROFT, J. Features in the architecture of physiological function. Cambridge, 1934.
(24) BARRON, E. S. G. J. Biol. Chem. **81**: 445; **84**: 83, 1929.
J. Exper. Med. **52**: 447, 1930.
(25) BARRON, E. S. G. J. Biol. Chem. **97**: 287, 1932.
(26) BARRON, E. S. G. J. Biol. Chem. **121**: 285, 1937.
(27) BARRON, E. S. G. J. Biol. Chem. Proc. **119**: vi, 1937.
(28) BARRON, E. S. G., A. G. BARRON AND F. KLEMPERER. J. Biol. Chem. **116**: 563, 1936.
(29) BARRON, E. S. G., R. H. DEMEIO AND F. KLEMPERER. J. Biol. Chem. **112**: 625, 1936.
(30) BARRON, E. S. G., L. B. FLEXNER AND L. MICHAELIS. J. Biol. Chem. **81**: 743, 1929.
(31) BARRON, E. S. G. AND G. A. HARROP, JR. J. Biol. Chem. **79**: 65, 1928.
(32) BARRON, E. S. G. AND A. B. HASTINGS. J. Biol. Chem. **100**: 155, 1933.
(33) BARRON, E. S. G. AND A. B. HASTINGS. J. Biol. Chem. **107**: 567, 1934.
(34) BARRON, E. S. G. AND L. A. HOFFMAN. J. Gen. Physiol. **13**: 483, 1930.
(35) BARRON, E. S. G. AND H. JACOBS. J. Bacteriol. **36**: 433, 1938.
(36) BARRON, E. S. G. AND C. M. LYMAN. J. Biol. Chem. **123**: 229, 1938.
(37) BARRON, E. S. G. AND C. M. LYMAN. J. Biol. Chem. **127**: 143, 1939.
(38) BARRON, E. S. G. AND P. C. MILLER, JR. J. Biol. Chem. **97**: 691, 1932.
(39) BAUR, E. AND A. GLAESSNER. Ztschr. Elektrochem. **9**: 534, 1903.
(40) BERNHEIM, F. AND M. L. C. BERNHEIM. J. Biol. Chem. **96**: 325, 1932; **109**: 131, 1935.
(41) BLASHKO, H., D. RICHTER AND H. SCHLOSSMANN. J. Physiol. **90**: 1, 1937.
(42) BOOTH, V. H. Biochem. J. **32**: 494, 1938.
(43) BORSOOK, H. Ergebn. Enzymforsch. **4**: 1, 1935.
(44) BORSOOK, H., H. W. DAVENPORT AND C. E. P. JEFFREYS. J. Biol. Chem. **117**: 237, 1937.

(45) Borsook, H., E. L. Ellis and H. M. Huffman. J. Biol. Chem. **117**: 281, 1937.
(46) Borsook, H. and G. Keighley. Proc. Nat. Acad. Sc. **19**: 875, 1933.
(47) Borsook, H. and H. F. Schott. J. Biol. Chem. **92**: 535, 1931.
(48) Boyland, E. and M. E. Boyland. Biochem. J. **30**: 877, 1936.
(49) Boysen-Jensen, P. Biochem. Ztschr. **236**: 211, 1931.
(50) Braunstein, A. E. and M. G. Kritzmann. Biokhimiya **2**: 242, 1937.
(51) Breusch, F. L. Ztschr. physiol. Chem. **250**: 262, 1937.
(52) Brönsted, J. N. Chem. Rev. **5**: 231, 1928.
(53) Bumm, E., H. Appel and K. Fehrenbach. Ztschr. physiol. Chem. **223**: 207, 1934.
(54) Burk, D. Some fundamental aspects of the cancer problem. Science Press, 1937.
(55) Bushmakin, I. N. and K. I. Kuchinskaya. Trudy Gosud. Opyt. Zavoda Sint. Kauchuka, Litera B. Synthetic Rubber **4**: 160, 1935.
(56) Cannan, R. K. Biochem. J. **20**: 927, 1926.
(57) Cannan, R. K. Biochem. J. **21**: 184, 1927.
(58) Chang, T. H., R. W. Gerard and M. Shaffer. Am. J. Physiol. **101**: 19, 1932.
(59) Chapheau, M. Bull. Stat. Biol. Arcachon **27**: 29, 1930.
(60) Chow, B. F. J. Am. Chem. Soc. **57**: 1437, 1935.
(61) Chow, B. F. and S. E. Kamerling. J. Biol. Chem. **104**: 69, 1934.
(62) Clark, W. M. Pub. Health Repts. **38**: 443, 1923.
(63) Clark, W. M. Medicine **13**: 207, 1934.
(64) Clark, W. M. J. Applied Physics **9**: 97, 1938.
(65) Clark, W. M. and B. Cohen. Pub. Health Repts. **38**: 666, 1923.
(66) Clark, W. M., B. Cohen and H. D. Gibbs. Pub. Health Repts. **40**: 1131, 1925.
(67) Clark, W. M., J. F. Taylor, T. H. Davies and R. Lewis. Compt. Rend. Lab. Carlsberg Sér. Chim. **22**: 129, 1938.
(68) Cohen, E. and C. A. Elvehjem. J. Biol. Chem. **107**: 97, 1934.
(69) Cohen, R. A. and R. W. Gerard. J. Cell. and Comp. Physiol. **10**: 223, 1937.
(70) Conant, J. B. Chem. Rev. **3**: 1, 1926.
(71) Conant, J. B., G. A. Alles and C. O. Tongberg. J. Biol. Chem. **79**: 89, 1928.
(72) Conant, J. B. and C. O. Tongberg. J. Biol. Chem. **86**: 733, 1930.
(73) Coolidge, T. B. J. Biol. Chem. **98**: 755, 1932.
(74) Corran, H. S. and D. E. Green. Nature **142**: 149, 1938.
(75) Das, N. B. Ztschr. physiol. Chem. **238**: 269, 1936.
(75a) Davidson, J. N. Biochem. J. **32**: 1386, 1938.
(76) De Meio, R. H., M. Kissin and E. S. G. Barron. J. Biol. Chem. **107**: 579, 1934.
(77) Dewan, J. G. and D. E. Green. Nature **140**: 1097, 1937.
(78) Dewan, J. G. and D. E. Green. Biochem. J. **31**: 1074, 1937.
(79) Dickens, F. Biochem. J. **28**: 537, 1934; **30**: 1323, 1936.
(80) Dilling, W. J. Atlas der Kristallformen und der Absorptionsbänder der Hämochromogene. Stuttgart, 1910.

(81) DIMROTH, O. Ztschr. angew. Chem. 46: 571, 1933.
(82) DIXON, K. C. Biochem. J. 30: 1483, 1936.
(83) DIXON, K. C. Biol. Rev. 12: 431, 1937.
(84) DIXON, M. Proc. Roy. Soc. (London) B101: 57, 1927.
(85) DIXON, M. AND K. A. C. ELLIOTT. Biochem. J. 23: 812, 1929.
(86) DIXON, M. AND J. H. QUASTEL. J. Chem. Soc. 123: 2943, 1923.
(87) DOBSON, H. J. E. J. Chem. Soc. 127: 2866, 1925.
(88) DRABKIN, D. L. J. Biol. Chem. Proc. 123: xxxi, 1938.
(89) ELEMA, B. Rec. Trav. Chim. Pays Bas 50: 807, 1931.
(90) ELEMA, B. Rec. Trav. Chim. Pays Bas 52: 569, 1933.
(91) ELLIOTT, K. A. C., M. E. GREIG AND M. P. BENOY. Biochem. J. 31: 1003, 1937.
(92) ELLIOTT, K. A. C. AND M. E. GREIG. Biochem. J. 31: 1021, 1937.
(93) ERLICH, P. Das Sauerstoffbedürfniss der Zelle. Berlin, 1883.
(94) EULER, H. v. Ergebn. Physiol. 38: 1, 1936.
(95) EULER, H. v. Ztschr. angew. Chem. 50: 831, 1937.
(96) EULER, H. v. AND E. ADLER. Ztschr. physiol. Chem. 223: 105, 1934.
(97) EULER, H. v. AND E. ADLER. Ztschr. physiol. Chem. 252: 41, 1938.
(98) EULER, H. v., E. ADLER AND T. S. ERIKSEN. Ztschr. physiol. Chem. 248: 227, 1937.
(99) EULER, H. v., E. ADLER AND G. GÜNTHER. Ztschr. physiol. Chem. 249: 1, 1937.
(100) EULER, H. v., E. ADLER, G. GÜNTHER AND H. HELLSTRÖM. Ztschr. physiol. Chem. 245: 217, 1937.
(100a) EULER, H. v., E. ADLER, G. GÜNTHER AND N. B. DAS. Ztschr. physiol. Chem. 254: 61, 1938.
(101) EULER, H. v. AND P. KARRER. Helv. chim. acta 19: 1060, 1936.
(102) EULER, H. v. AND F. SCHLENK. Ztschr. physiol. Chem. 246: 64, 1937.
(103) EULER, H. v. AND R. VESTIN. Naturwiss. 25: 416, 1937.
(104) EYRING, J. J. Chem. Phys. 3: 107, 1935.
(105) FIESER, L. B. J. Am. Chem. Soc. 52: 4915, 1930.
(106) FILITTI, S. J. Chim. Phys. 32: 1, 1935.
(107) FISCHER, H. AND F. KÖGEL. Ztschr. physiol. Chem. 138: 269, 1924.
(108) FISHBERG, H. AND B. T. DOLIN. J. Biol. Chem. 101: 159, 1933.
(109) FLEISCH, A. Biochem. J. 17: 294, 1924.
(110) FLEISCHMANN, W. AND S. KANN. Biochem. Ztschr. 257: 293, 1933.
(111) *FREI, W., L. RIEDMÜLLER AND F. ALMASY. Biochem. Ztschr.* 274: 253, 1934.
(112) FRIEDHEIM, E. A. H. Compt. Rend. Soc. Biol. 110: 353, 1932.
(113) FRIEDHEIM, E. A. H. Compt. Rend. Soc. Biol. 112: 1030, 1933.
(114) FRIEDHEIM, E. A. H. Biochem. Ztschr. 259: 257, 1933.
(115) FRIEDHEIM, E. A. H. Biochem. J. 28: 173, 1934.
(116) FRIEDHEIM, E. A. H. Biochem. J. 28: 180, 1934.
(117) FRIEDHEIM, E. A. H. AND L. MICHAELIS. J. Biol. Chem. 91: 355, 1931.
(118) FRUTON, J. S. J. Biol. Chem. 105: 79, 1934.
(119) FRUTON, J. S. AND H. T. CLARKE. J. Biol. Chem. 106: 667, 1934.
(120) FUJITA, A. AND T. KODAMA. Biochem. Ztschr. 277: 17, 1935.
(121) GEORGESCU, I. D. J. Chim. Phys. 29: 217, 1932.
(122) GERARD, R. W. Proc. Soc. Exper. Biol. and Med. 27: 1052, 1930.

(123) GERARD, R. W. Am. J. Physiol. 97: 523, 1931.
(124) GERARD, R. W. Physiol. Rev. 12: 469, 1932.
(125) GERARD, R. W. AND L. H. HYMAN. Am. J. Physiol. 97: 524, 1931.
(126) GERSCHINOWITZ, H. J. Chem. Phys. 4: 363, 1936.
(127) GHOSH, J. C. AND S. C. GANGULI. Biochem. J. 28: 381, 1934.
(128) GHOSH, J. C., S. N. RAYCHANDRI AND S. C. GANGULI. J. Ind. Chem. Soc. 9: 43, 1932.
(129) GILLESPIE, L. J. Soil Science 9: 199, 1920.
(130) GILLESPIE, L. J. AND T. H. LIU. J. Am. Chem. Soc. 53: 3969, 1931.
(131) GREEN, D. E. Biochem. J. 27: 678, 1933.
(132) GREEN, D. E. Biochem. J. 27: 1044, 1933.
(133) GREEN, D. E. Proc. Roy. Soc. (London) B114: 423, 1934.
(134) GREEN, D. E. Biochem. J. 28: 1550, 1934.
(135) GREEN, D. E. AND J. BROSTREAUX. Biochem. J. 30: 1489, 1936.
(136) GREEN, D. E. AND J. G. DEWAN. Biochem. J. 31: 1069, 1937.
(137) GREEN, D. E. AND J. G. DEWAN. Biochem. J. 32: 1200, 1938.
(138) GREEN, D. E., J. C. DEWAN AND L. F. LELOIR. Biochem. J. 31: 934, 1937.
(139) GREEN, D. E. AND D. RICHTER. Biochem. J. 31: 596, 1937.
(140) GREEN, D. E., L. H. STICKLAND AND H. L. A. TARR. Biochem. J. 28: 1812, 1934.
(141) GREVILLE, G. D. Biochem. J. 30: 877, 1936.
(142) GRUBE, G. AND K. HUBERICH. Ztschr. Elektrochem. 29: 8, 1923.
(143) GYORGYI, P., R. KUHN AND TH. WAGNER-JAUREGG. Ztschr. physiol. Chem. 223: 21, 1934.
(144) HAAS, E. Naturwiss. 22: 207, 1934.
(145) HAAS, E. Biochem. Ztschr. 290: 291, 1937.
(146) HAMMETT, L. P. Chem. Rev. 17: 125, 1935.
(147) HARRINGTON, C. R. AND T. H. MEAD. Biochem. J. 29: 1602, 1935.
(148) HARRISON, D. C. Biochem. J. 27: 382, 1933.
(149) HARROP, G. A., JR. AND E. S. G. BARRON. J. Exper. Med. 48: 207, 1928.
(150) HELLERMAN, L. Physiol. Rev. 17: 454, 1937.
(151) HELLSTRÖM, H. Ztschr. physiol. Chem. 246: 155, 1937.
(152) HILL, E. S. Proc. Soc. Exper. Biol. and Med. 35: 363, 1936.
(152a) HILL, E. S. J. Amer. Chem. Soc. 60: 1990, 1938.
(153) HILL, R. Proc. Roy. Soc. (London) B105: 112, 1929.
(154) HINSHELWOOD, C. N. The kinetics of chemical changes in gaseous systems. 2nd ed., Oxford, 1929.
(155) HODEL, A. Biochem. Ztschr. 268: 285, 1934.
(156) HOGNESS, T. R., F. P. ZSCHEILE, JR., A. E. SIDWELL AND E. S. G. BARRON. J. Biol. Chem. 118: 1, 1937.
(157) HOPKINS, F. G. J. Biol. Chem. 84: 269, 1929.
(158) HOPKINS, F. G. AND K. A. C. ELLIOTT. Proc. Roy. Soc. (London) B109: 58, 1931.
(159) HOPKINS, F. G. AND E. G. MORGAN. Biochem. J. 32: 611, 1938.
(160) HUSZÁK, ST. Biochem. Ztschr. 298: 137, 1938.
(161) KARRER, P. AND F. BENZ. Helv. chim. acta 19: 1028, 1936.
(162) KARRER, P., G. SCHWARZENBACH, F. BENZ AND U. SOHNSSEN. Helv. chim. acta 19: 811, 1936.

(163) KARRER, P., G. SCHWARZENBACH AND K. SCHÖPP. Helv. chim. acta 16: 302, 1933.
(164) KARRER, P. AND F. J. STARE. Helv. chim. acta 20: 418, 1937.
(165) KARRER, P. AND O. WARBURG. Biochem. Ztschr. 285: 297, 1936.
(166) KEILIN, D. Proc. Roy. Soc. (London) B98: 312, 1925.
(167) KEILIN, D. Proc. Roy. Soc. (London) B100: 129, 1926; 104: 206, 1929.
(168) KEILIN, D. Ergebn. Enzymforsch. 2: 239, 1933.
(169) KEILIN, D. Bull. Soc. Chim. Biol. 17: 96, 1936.
(170) KEILIN, D. AND E. F. HARTREE. Proc. Roy. Soc. (London) B119: 114, 1936.
(171) KEILIN, D. AND E. F. HARTREE. Proc. Roy. Soc. (London) B121: 173, 1936.
(172) KEILIN, D. AND E. F. HARTREE. Proc. Roy. Soc. (London) B122: 298, 1937.
(173) KEILIN, D. AND E. F. HARTREE. Proc. Roy. Soc. (London) B125: 171, 1938.
(174) KEILIN, D. AND E. F. HARTREE. Nature 141: 870, 1938.
(175) KEILIN, D. AND T. MANN. Proc. Roy. Soc. (London) B122: 119, 1937.
(176) KEILIN, D. AND T. MANN. Proc. Roy. Soc. (London) B125: 187, 1938.
(177) KEKWICK, R. A. AND K. O. PEDERSEN. Biochem. J. 30: 2201, 1936.
(178) KEMPNER, W. Plant Physiol. 11: 605, 1936.
(179) KEMPNER, W. J. Cell. and Comp. Physiol. 10: 339, 1937.
(180) KENDALL, E. C. J. Biol. Chem. 84: 657, 1929.
(181) KING, C. G. Physiol. Rev. 16: 238, 1936.
(182) KISCH, B. Biochem. Ztschr. 242: 1, 1931.
(183) KISCH, B. AND J. LEIBOWITZ. Biochem. Ztschr. 220: 370, 1930.
(184) KISCH, B. AND K. SCHUWIRTH. Biochem. Ztschr. 247: 371, 1932; 257: 89, 1933.
(185) KLEMPERER, F., O. A. BESSEY AND A. B. HASTINGS. Proc. Soc. Exper. Biol. and Med. 37: 114, 1937.
(185a) KNOOP, F. Ztschr. physiol. Chem. 67: 489, 1910.
(186) KRAH, H. Biochem. Ztschr. 219: 432, 1930.
(187) KREBS, H. A. Biochem. Ztschr. 204: 322, 1929.
(188) KREBS, H. A. Ztschr. physiol. Chem. 217: 351, 1933.
(189) KREBS, H. A. Biochem. J. 29: 1620, 1935.
(190) KREBS, H. A. Biochem. J. 31: 645; 661, 1937.
(191) KREBS, H. A. AND W. A. JOHNSON. Biochem. J. 31: 772, 1937.
(192) KREBS, H. A. AND W. A. JOHNSON. Enzymologia 4: 148, 1937.
(193) KUBOWITZ, F. Biochem. Ztschr. 204: 475, 1929.
(194) KUBOWITZ, F. Biochem. Ztschr. 292: 221; 293: 308, 1937.
(195) KUHN, R. AND P. BOULANGER. Ber. Chem. Ges. 69: 1557, 1936.
(196) KUHN, R., D. B. HAND AND M. FLORKIN. Ztschr. physiol. Chem. 201: 255, 1931.
(197) KUHN, R. AND G. MORUZZI. Ber. chem. Ges. 67: 1220, 1934.
(198) KUHN, R., RUDY AND F. WEIGAND. Ber. chem. Ges. 69: 2034, 1936.
(199) KUHN, R. AND TH. WAGNER-JAUREGG. Ber. chem. Ges. 67: 361, 1934.
(200) KUHN, R., TH. WAGNER-JAUREGG AND H. KALTSCHMITT. Ber. chem. Ges. 67: 1452, 1934.
(201) LAKI, K. Ztschr. physiol. Chem. 217: 54, 1933.
(202) LAKI, K. Ztschr. physiol. Chem. 249: 61, 1937.
(203) LAKI, K. Ztschr. physiol. Chem. 249: 63, 1937.
(204) LA MER, V. K. J. Chem. Phys. 1: 289, 1933.

(205) La Mer, V. K. and J. W. Temple. Proc. Nat. Acad. Sc. **15:** 91, 1929.
(206) Langenbeck, W. Ber. chem. Ges. **65:** 842, 1932.
(207) Lavoisier, A. E. Expériences sur la respiration des animaux et sur les changements qui arrivent à l' air en passant par les poumons. Mem. de l' Acad. des Sc. Paris, 1777.
(208) Lehmann, J. Skand. Arch. Physiol. **58:** 173, 1929-30.
(209) Lehmann, J. Biochem. Ztschr. **274:** 321, 1934.
(210) Leiner, G. Biochem. Ztschr. **276:** 186, 1935.
(211) Leloir, L. F. and M. Dixon. Enzymologia **2:** 81, 1937.
(212) Lewis, G. N. and M. Randall. Thermodynamics and the free energy of chemical substances. New York, 1923.
(213) Lipmann, F. Biochem. Ztschr. **265:** 133, 1935.
(214) Lipmann, F. Enzymologia **4:** 65, 1937.
(215) Lipmann, F. and G. Perlmann. J. Am. Chem. Soc. **60:** 2574, 1938.
(216) Lipschitz, M. E., V. R. Potter and C. A. Elvehjem. Biochem. J. **32:** 1474, 1938.
(217) Lohmann, K. and Ph. Schuster. Biochem. Ztschr. **294:** 186, 1937.
(218) Lundsgaard, E. Biochem. Ztschr. **217:** 162, 1930; **220:** 8, 1932.
(219) Lyman, C. M. and E. S. G. Barron. J. Biol. Chem. **121:** 275, 1937.
(220) MacMunn, C. A. Phil. Transac. **177:** 267, 1886.
(221) Martius, C. and F. Knoop. Ztschr. physiol. Chem. **242:** 1, 1936; **246:** 1, 1937.
(222) Meldrum, N. U. and M. Dixon. Biochem. J. **24:** 472, 1930.
(223) Meyerhof, O. Pflüger's Arch. **169:** 87, 1917.
(224) Meyerhof, O. Ergebn. Physiol. **39:** 10, 1937.
(225) Meyerhof, O. and P. Ohlmeyer. Biochem. Ztschr. **290:** 334, 1937.
(226) Meyerhof, O. and W. Kiessling. Biochem. Ztschr. **264:** 40, 1933.
(227) Michaelis, L. Oxydations-Reductions-Potentiale. Zweite Auflage, Berlin, 1933.
(228) Michaelis, L. Chem. Rev. **16:** 243, 1935.
(229) Michaelis, L. Trans. Electrochem. Soc. **71:** 107, 1937.
(230) Michaelis, L. and M. P. Schubert. Chem. Rev. **22:** 437, 1938.
(231) Michaelis, L. and L. B. Flexner. J. Biol. Chem. **79:** 689, 1928.
(232) Michaelis, L., M. P. Schubert and C. V. Smythe. J. Biol. Chem. **116:** 587, 1936.
(233) Michaelis, L. and C. V. Smythe. J. Biol. Chem. **113:** 717, 1936.
(234) Millikan, G. A. Proc. Roy. Soc. (London) **B120:** 366, 1936.
(235) Mori, P., K. Okumuki and E. Yakushiji. Acta Phytochim. (Japan) **10:** 81, 1937.
(236) Müller, D. Biochem. Ztschr. **199:** 136, 1928.
(237) Myrback, K. Ergebn. Enzymforsch. **2:** 139, 1933.
(238) Nakashima, T. Biochem. Ztschr. **204:** 479, 1929.
(239) Needham, D. M. and R. K. Pillai. Biochem. J. **41:** 1837, 1937.
(240) Negelein, E. and W. Gerischer. Biochem. Ztschr. **284:** 289, 1936.
(241) Negelein, E. and E. Haas. Biochem. Ztschr. **282:** 206, 1935.
(242) Negelein, E. and H. J. Wulff. Biochem. Ztschr. **289:** 436; **293:** 351, 1937.
(243) Ochoa, S. Biochem. Ztschr. **292:** 68, 1937.
(244) Oparin, R. Biochem. Ztschr. **124:** 90, 1921; **182:** 155, 1927.

(245) PALLADIN, W. Biochem. Ztschr. 27: 442, 1910.
(246) PARKS, G. S. AND H. M. HUFFMAN. The free energies of some organic compounds. New York, 1932.
(247) PASTEUR, L. Bull. Soc. Chim. Paris, P. 79, 1861.
(248) PAULING, L. AND C. D. CORYEL. Proc. Nat. Acad. Sci. 22: 210, 1936.
(249) PEDERSEN, K. J. J. Phys. Chem. 38: 601, 1934.
(250) PETERS, R. A. Chem. Weekblad 34: no. 26, 1937.
(251) PREISLER, P. W. J. Gen. Physiol. 13: 349, 1930.
(252) PREISLER, P. W. AND P. A. SHAFFER. Am. Chem. Soc. Meetings. Chicago, 1933.
(253) QUASTEL, J. H. AND M. D. WHETHAM. Biochem. J. 18: 519, 1924.
(254) QUASTEL, J. H. AND W. R. WOOLDRIDGE. Biochem. J. 22: 689, 1928.
(255) RAPKINE, L. Compt. Rend. Acad. Sc. 207: 301, 1938.
(256) REID, A. Ergebn. Enzymforsch. 1: 325, 1932.
(257) RICE, O. K. J. Chem. Phys. 4: 53, 1936.
(258) RICE, O. K. AND H. GERSCHINOWITZ. J. Chem. Phys. 2: 853, 1934.
(259) ROCHE, J. Les pigments hematiniques des actinies et l'actinohematine. Contribution à l'étude des cytochromes. Lons-Le-Saunier, 1936.
(260) ROCHE, J. AND M. T. BENEVENT. Bull. soc. chim. biol. 18: 1650, 1936.
(261) RUDY, H. Ztschr. physiol. Chem. 239: 47, 1936.
(262) SATO, T. AND H. TAMIYA. Cytologia 8: 1133, 1937.
(263) SCHLENK, F. Naturwiss. 25: 668, 1937.
(264) SCHLENK, F., H. HELLSTRÖM AND H. V. EULER. Ber. chem. Ges. 71: 1471, 1938.
(265) SHAFFER, P. A. J. Am. Chem. Soc. 55: 2169, 1933.
(266) SHAFFER, P. A. J. Phys. Chem. 40: 1021, 1936.
(267) SHERRILL, M. S. AND A. J. HAAS, JR. J. Am. Chem. Soc. 58: 952, 1936.
(268) SHIBATA, K. Ergebn. Enzymforsch. 4: 348, 1935.
(269) SHORR, E., S. B. BARKER AND M. MALANI. Science 87: 168, 1937.
(270) SIDWELL, A. E., JR., R. H. MUNCH, E. S. G. BARRON AND T. R. HOGNESS. J. Biol. Chem. 123: 335, 1938.
(271) STARE, F. I. AND G. A. BAUMAN. Proc. Roy. Soc. (London) B121: 338, 1936.
(272) STARE, F. I. J. Biol. Chem. 112: 223, 1935–36.
(273) STARE, F. I. Biochem. J. 30: 2257, 1936.
(274) STERN, K. G. Biochem. J. 28: 949, 1934.
(275) STERN, K. G. Biochem. J. 29: 500, 1935.
(276) STERN, K. G. J. Biol. Chem. 114: 473, 1936.
(277) STERN, K. G. J. Biol. Chem. 121: 561, 1937.
(278) STOTZ, E., C. J. HARRER AND C. G. KING. J. Biol. Chem. 119: 511, 1937.
(279) STOTZ, E. AND A. B. HASTINGS. J. Biol. Chem. 118: 479, 1937.
(280) STOTZ, E., A. E. SIDWELL AND T. R. HOGNESS. J. Biol. Chem. 124: 11, 1938.
(281) SUMNER, J. B. AND A. L. DOUNCE. J. Biol. Chem. 121: 417, 1937.
(282) SZENT-GYÖRGYI, A. Biochem. Ztschr. 150: 194, 1924.
(283) SZENT-GYÖRGYI, A. Perspectives in biochemistry. Cambridge, 1937.
(284) SZENT-GYÖRGYI, A. Studies on biological oxidation and some of its catalysts. Leipzig, 1937.
(285) TAMIYA, H. AND S. YAMAGUTCHI. Acta Phytochim. 7: 233, 1933.

(286) Tauber, H. Enzymologia 2: 17, 1937.
(287) Tauber, H. Ergebn. Enzymforsch. 7: 301, 1938.
(288) Tauber, H. J. Am. Chem. Soc. 60: 730, 1938.
(289) Theorell, H. Biochem. Ztschr. 285: 207, 1936.
(290) Theorell, H. Biochem. Ztschr. 288: 317, 1936.
(291) Theorell, H. Biochem. Ztschr. 290: 293, 1937.
(292) Theorell, H. Ergebn. Enzymforsch. 6: 111, 1937.
(293) Theorell, H. Biochem. Ztschr. 298: 242, 1938.
(294) Thunberg, T. Skand. Arch. Physiol. 46: 339, 1925.
(295) Thunberg, T. Ergebn. Enzymforsch. 7: 163, 1938.
(296) Treibs, A. Ztschr. physiol. Chem. 212: 26, 1932.
(297) Verzar, F., H. Hübner and L. Lastz. Biochem. Ztschr. 292: 152, 1937.
(298) Vestin, R. Naturwiss. 25: 667, 1937.
(298a) du Vigneaud, V. and O. J. Irish. J. Biol. Chem. 122: 349, 1938.
(299) Wagner-Jauregg, Th. and E. F. Möller. Ztschr. physiol. Chem. 236: 222, 1935.
(300) Wagner-Jauregg, Th. Handbuch der biologischen Arbeitsmethoden. V Teil 3 B, p. 1211. Berlin, 1937.
(301) Warburg, O. Biochem. Ztschr. 172: 432, 1926.
(302) Warburg, O. Biochem. Ztschr. 189: 354, 1927.
(303) Warburg, O. Ztschr. angew. Chem. 45: 1, 1932.
(304) Warburg, O. Ergebn. Enzymforsch. 7: 210, 1938.
(305) Warburg, O. and W. Christian. Naturwiss. 20: 980, 1932.
(306) Warburg, O. and W. Christian. Biochem. Ztschr. 260: 499, 1933.
(307) Warburg, O. and W. Christian. Biochem. Ztschr. 266: 377, 1933.
(308) Warburg, O. and W. Christian. Biochem. Ztschr. 275: 464, 1934.
(309) Warburg, O. and W. Christian. Biochem. Ztschr. 292: 287, 1937.
(310) Warburg, O. and W. Christian. Biochem. Ztschr. 295: 261, 1938.
(311) Warburg, O. and W. Christian. Biochem. Ztschr. 298: 150, 1938.
(312) Warburg, O., W. Christian and A. Griese. Biochem. Ztschr. 279: 143, 1935.
(313) Warburg, O. and F. Kubowitz. Biochem. Ztschr. 203: 95, 1928.
(314) Warburg, O., F. Kubowitz and W. Christian. Biochem. Ztschr. 221: 494; 227: 245, 1930.
(315) Warburg, O. and E. Negelein. Biochem. Ztschr. 193: 339, 1928.
(316) Warburg, O. and E. Negelein. Biochem. Ztschr. 214: 101, 1929.
(317) Warburg, O., E. Negelein and E. Haas. Biochem. Ztschr. 227: 171, 1930.
(318) Warburg, O. and S. Sakuma. Pflüger's Arch. 200: 203, 1923.
(319) Weill-Malherbe, H. Biochem. J. 31: 2202, 1937.
(320) Wendel, W. B. Proc. Soc. Exper. Biol. Med. 27: 624; 28: 401, 1930.
(321) Wieland, H. On the mechanism of oxidation. New Haven, 1932.
(322) Wieland, H. and T. F. Macrae. Ann. der. Chem. 483: 217, 1930.
(323) Williams, J. W. and E. M. Drissen. J. Biol. Chem. 87: 441, 1930.
(324) Williams, R. R. and T. D. Spies. Vitamin B_1. New York, 1938.
(325) Wishart, G. M. Biochem. J. 17: 103, 1923.
(326) Woods, D. D. Biochem. J. 30: 515, 1936.
(327) Wurmser, R. and S. Filitti-Wurmser. J. Chim. Phys. 33: 577, 1936.

(328) Wurmser, R. and S. Filitti-Wurmser. Compt. Rend. Soc. Biol. **127**: 471, 1938; J. Chim. Phys. **35**: 81, 1938.
(329) Wurmser, R. and S. Filitti-Wurmser. Compt. Rend. Soc. Biol. **128**: 133, 1938.
(330) Wurmser, R. and J. A. de Loureiro. J. Chim. Phys. **31**: 419, 1934.
(331) Wurmser, R. and N. Mayer-Reich. J. Chim. Phys. **30**: 249, 1933.
(332) Yakushiji, E. and T. Mori. Acta Phytochim. (Japan) **10**: 113, 1937.
(333) Yamagutchi, S. Acta Phytochim. (Japan) **10**: 171, 1937.
(334) Yamagutchi, S. Botan. Mag. **51**: 275, 1937.
(335) Yaoi, H. and H. Tamiya. Proc. Imp. Acad. Tokyo **4**: 436, 1928.
(336) Zeile, K. and H. Hellström. Ztschr. physiol. Chem. **192**: 171, 1930; **195**: 39, 1931.

BACTERIAL CHEMOTHERAPY

The Pharmacology of Sulfanilamide

E. K. MARSHALL, JR.

The Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University

The idea of developing specific chemical substances for the treatment of bacterial infections is by no means a new one; in fact, it is as old as bacteriology itself. In 1881, Koch (97) attempted to cure guinea pigs infected with anthrax by the injection of mercuric salts. Although an amount of mercury was used which was more than sufficient, on the assumption of its equal distribution in the host, to destroy the bacteria *in vitro*, the results were not only entirely negative, but the treatment appeared to shorten the life of the infected animal. These experiments were so discouraging that many believed it would be impossible to find any specific for the infectious diseases. Only in 1904, when Ehrlich's studies (52) in chemotherapy began to yield definite results, did interest in the search for drugs as bacterial chemotherapeutic agents revive. However, despite the advances which have been made since Ehrlich's time in the chemotherapy of protozoan infections, it is probably correct to say that nothing of any practical importance was developed in bacterial chemotherapy until within the last few years when drugs containing the sulfonamide group were introduced.[1] This failure was not due to the lack of experimentation along these lines, but probably to the fact that such investigation is still in the stage of haphazard trial and error. It may be recalled that Morgenroth and Levy's use (139) in 1911 of ethylhydrocupreine to cure pneumococcus septicemia in mice was practically the only instance known of an experimental bacterial infection in animals which could be cured by a drug. Moreover, the

[1] For a discussion of earlier work in this field, the reviews of Dale (40) and Jacobs (91) and the book by Findlay (57) can be consulted. Reference must be made to the more recent work of Feldt (56) and Collier (35) on the use of organic gold compounds in pneumococcus and streptococcus infections in mice, to the work of Browning and his colleagues (15) on anilquinoline compounds, and to the recent work on new quinine derivatives (145, 92, 119).

curative dose was very near the lethal dose, and this remedy when tried in pneumonia in man was found too toxic to be given in sufficient dosage for effective therapy.

The above situation has been radically altered by the discovery of the sulfonamide derivatives as bacterial chemotherapeutic agents. The known scope of effective treatment with these compounds is widening rapidly, and while brilliant success has already been achieved, so much ground remains to be explored that the ultimate consequences of this discovery are beyond prediction. We can only trace briefly the development of this important subject. Gelmo (69) in 1908 in an investigation of a purely scientific nature first described the preparation of the compound, para-aminobenzenesulfonamide (sulfanilamide). A year later, Hörlein, Dressel and Kothe of the I. G. Farbenindustrie prepared the first azo dyes with sulfonamide and substituted sulfonamide groups, and found they were distinguished by greater fastness to washing and milling than those of the corresponding sulfonamide-free products. Eisenberg (53) demonstrated the bactericidal action of certain azo compounds *in vitro* and employed chrysoidine for chemotherapeutic purposes, but not the slightest therapeutic effect could be obtained in infected animals. Heidelberger and Jacobs (85) tried to increase the bactericidal properties of the quinine molecule by linking dihydrocupreine with diazo compounds obtained from aromatic amines, including para-aminobenzenesulfonamide, and in their article they state that many of the azo compounds thus formed exhibit bactericidal properties *in vitro* (like the azo dyes of Eisenberg).

In the laboratories of the I. G. Farbenindustrie at Elberfeld, a series of investigations led to azo compounds with marked bactericidal properties *in vitro* but no action *in vivo* in the mouse. In studies confined to compounds containing the sulfonamide group and tested only by animal experiment, drugs effective against streptococcus infections were discovered (47, 89, 135). In 1932, Mietzsch and Klarer obtained a patent for the azo dye, sulfamidochrysoidine (4-sulfamido-2′:4′-diaminoazobenzene), which was found by Domagk (48) to cure an otherwise fatal streptococcus infection in mice. This compound was put out under the trade name of "Prontosil." A more soluble compound, to be given by intramuscular injection, was described shortly after the appearance of the first product (46). This compound is the sodium salt of 4′-sulfonamidophenyl-2-azo-7-acetyl-amino-1-hydroxynaphthylene-3-,6-disulfonic acid and was called "Prontosil Soluble" and later "Neoprontosil." Levaditi and Vaisman (108), using sulfamido-

chrysoidine, confirmed in the main the findings of Domagk, as also did Nitti and Bovet (141). Tréfouel, Tréfouel, Nitti and Bovet (171) found that a number of azo dyes prepared from para-amino-benzenesulfonamide but of different physical and chemical properties had an anti-streptococcus action like "Prontosil"; they found, however, that if other aromatic amines were used in preparing the azo dyes they were inactive therapeutically. On the basis of these facts they proposed the hypothesis that all the active azo dyes, including "Prontosil," were reduced in the organism to sulfanilamide. This led them to the very important discovery that the simple colorless compound, sulfanilamide, was itself highly active as a therapeutic agent in experimental streptococcus infections. Colebrook and Kenny (34) and Buttle, Gray and Stephenson (20) in England confirmed the fact that both the prontosils and sulfanilamide were effective against experimental streptococcus infections in mice, as did Long and Bliss (114) in America.

Even before the appearance of Domagk's paper in 1935, mention of the treatment with Streptozon (Prontosil) of patients with infections appeared in German clinical journals (62, 78, 79, 174, 73) and afterwards numerous favorable results in human infections were reported (164, 2, 163, 161, 65, 99) but it was only with the publication of the paper by Colebrook and Kenny (34) on the marked beneficial effect of the prontosils in puerperal sepsis that much interest was awakened in English-speaking countries in the new drugs.

Since sulfanilamide has been used, both in experimental investigations and in the treatment of infectious diseases in man, much more widely than any other of these newer compounds, since certain other compounds probably act by forming this substance in the host, and since more information is available in regard to its pharmacology, the purpose of the present review with its limited space can be best served by discussing mainly this compound and limiting the discussion of other compounds to such points as bear upon important problems of bacterial chemotherapy. We shall not attempt to deal with the clinical use of this drug or the many important problems that have arisen in this connection, but shall limit our discussion to the experimental results which form the basis for the intelligent use of sulfanilamide and related drugs in the therapy of certain bacterial diseases. In a study of the pharmacology of any chemotherapeutic agent three lines of investigation are important: namely, the effect of the drug on the infecting organism *in vivo* in animals, the effect of the drug on the infecting organism *in vitro* under various conditions, and the effect of the drug

on the host. We propose to discuss the treatment of experimental streptococcus and other infections in animals, the toxicity, absorption, excretion and distribution of sulfanilamide, its action on specific organs and tissues, the relation of chemical constitution to bacterial chemotherapeutic action, and the mechanism of action. The field is so new, however, that it is difficult in many instances to sift conflicting data, to assess poorly conducted experiments on small numbers of animals, or to be at all sure of the answer to many important questions which must furnish the basis for an understanding of the subject. Also, since many of the articles dealing with this problem are of the nature of preliminary reports, conclusions based on these papers may have to be modified in the near future.

Treatment of experimental streptococcus infections. Nearly all of the studies with sulfanilamide (and allied drugs) on experimental streptococcus infections have been on mice. Intraperitoneal injection of 0.2 to 0.5 cc. of a high dilution of a mouse-virulent culture of group A of β-hemolytic streptococci followed (or occasionally preceded) by the subcutaneous or per os administration of the drug with a repetition of the dose at varying intervals has been the usual procedure. Control animals usually die within 48 hours, while treated animals may survive much longer or recover from the infection.

Of the three main classes of streptococci, alpha, beta, and gamma (13), only organisms of the β-hemolytic streptococcus group produce satisfactory experimental infections in mice. Nothing therefore is known about the efficacy of sulfanilamide in alpha- and gamma-streptococcus infections in mice (10). In dogs with injured heart valves, a streptococcus viridans infection is stated to have been cured by sulfanilamide (96). According to Lancefield's classification, the β-hemolytic streptococci comprise nine groups designated by letters of the alphabet. In mice infected with mouse-virulent organisms of group A, composed almost exclusively of strains of human origin, sulfanilamide has been found to exhibit a pronounced therapeutic effect (34, 20, 114). According to Buttle, Gray and Stephenson (20) protection can be obtained with sulfanilamide against serologically different types of group A. Few experimental studies have been made on infections in mice with β-hemolytic streptococci belonging to groups other than A. Some protective value of sulfanilamide in mice infected with groups B and C organisms has been reported (10, 131, 166) but the results were not comparable to those obtained in mice infected with group A strains. These experiments are, however, so limited in scope that definite con-

clusions cannot be drawn. No data are available for infections in mice with other groups.

It was stated early that a necessary condition for successful therapy in mice was the use of a strain of hemolytic streptococci of high virulence. Few details of the experiments on which this conclusion is based have been given and most of the observations have been made with the original drug—"Prontosil." Nitti and Bovet (141) were the first to limit the action of the drug to mouse infections with highly virulent strains: their finding is confirmed by Colebrook and Kenny (34), Long and Bliss (114), Bloch-Michel, Conté and Durel (11), and Rosenthal (156). On the other hand, Levaditi and Vaisman (105), Gley and Girard (72), Bürgers (16), and Mellon, Gross and Cooper (132) found protection in mice against strains of low or moderate virulence. Since it is known that even with a highly mouse-virulent strain of streptococci protection with sulfanilamide may be obtained against 10,000 but not against 1,000,000 L.D. (20), and that *in vitro* a bacteriostatic or bactericidal action can be demonstrated only against a limited number of organisms, it would appear from the above results that the important factor in successful sulfanilamide therapy in mice is not the number of L.D. given, but the number of organisms used for infection. Dawson and Hobby (42) found that whereas protection cannot be obtained with a culture of low virulence when given in undiluted form, it can be obtained against the same strain when mucin is used to reduce the L.D. In addition, it is known that better therapeutic results are obtained with a culture of moderate than with one of high mouse virulence provided only small numbers of bacteria are injected.

An interesting and important observation from the standpoint of the mechanism of action of these drugs is the fact that treated mice may die from streptococcus septicemia a long time after treatment has ceased and without showing any symptoms in the interval. Thus, Levaditi and Vaisman (105) had mice dying 18 to 19 days after apparent cure of streptococcus infection, and Long and Bliss (113) report such delayed deaths even after 63 and 128 days.

Such factors as the strain of streptococcus, the size of infecting dose, the time after infection of commencing treatment, the dosage of sulfanilamide used, the interval between doses, and the length of the period of therapy appear to be of importance for the therapeutic effect obtained. The information available on these points is rather meagre and has been obtained usually on an insufficient number of mice to render the results conclusive. It is certain that repetition of a dose several times is much

more effective than a single dose (20, 55). Results can also be found to show that large doses given repeatedly are more effective than smaller ones (20, 23, 128), but no data are available to show what the optimal therapeutic concentration of sulfanilamide in a mouse's blood should be if maintained constant for several days. It is not yet certain whether a continuously maintained low concentration in the blood is more effective than repeated rises of concentrations to high levels with very little or no drug present in the intervals, but such data as exist would indicate that the best therapeutic effect results from the maintenance of a constant blood concentration for several days. The increase in effectiveness of large doses may be due to the longer maintenance of an effective blood concentration of the drug. Little can be said at present about the interval between doses; as stated above the evidence appears to indicate that a constant concentration of drug must be maintained in the blood and tissues for several days in order to obtain the maximum therapeutic effect. In most experiments on mice, the first dose of sulfanilamide has been given immediately after infection. There is good evidence, however, that the drug is still effective when the first dose is given several hours after infection and at a time when a positive blood culture is obtained (20, 114, 131), but due to the small numbers of mice used in most of these experiments, it is impossible at present to assess quantitatively the effect of delaying treatment.

Very few investigations have been concerned with the efficacy of sulfanilamide in experimental streptococcus infections in animals other than the mouse. Mention may be made of the use of the rat by Montestruc (137, 138), Meunier and Nitti (134) and Adolph and Lockwood (1), of the guinea pig by Mellon, Gross and Cooper (131), Seastone (166), and Habs and Bader (80), and of the rabbit by Domagk (48), Nitti and Bovet (142), Montestruc (137), Bürgers (16), Gay and Clark (68), Schaffer (160), and Kolmer, Raiziss and Rule (98). In general, one can conclude from the above studies that sulfanilamide is an effective therapeutic agent in β-hemolytic streptococcus infections in these animals. It is to be noted that in group C infections in the guinea pig and rabbit excellent therapeutic results are reported. Also, it is of interest to note that acute infections but not chronic ones can be successfully treated in the guinea pig (166, 80).

Treatment of other experimental bacterial infections. Although the prontosils and sulfanilamide were at first supposed to be specific against the β-hemolytic streptococcus, subsequent investigations have shown

that these drugs (or allied compounds) may be effective against infections due to several different genera of bacteria, at least one due to a protozoa, and possibly against certain virus infections. Definite evidence has been presented for the efficacy of sulfanilamide in experimental mouse infections due to meningococcus (20, 149, 158, 14, 109, 175, 55), gonococcus (110, 30, 31), E. typhi and paratyphi B (22), Clostridium welchii (112), Sonne strain of dysentery bacillus (18), and a staphylococcus (49, 18, 133, 55). Temporary protection only, with delay of death, but not recovery, was found for mice infected with S. aertrycke, Friedländer's bacillus, P. pseudotuberculosis and P. septica (22).

In regard to pneumococcus infection with types I, II and III in mice, there is agreement of opinion that the drug possesses some activity; different investigators do not agree, however, as to magnitude of the effect. Buttle, Gray and Stephenson (20), Buttle, Parish, McLeod and Stephenson (22), Bürgers (16), Long and Bliss (115), Whitby (175), Feinstone, Bliss, Ott and Long (55) report that although death may be delayed in treated mice, recovery does not occur. Rosenthal (156), Cooper, Gross and Mellon (37), and Fourneau, Tréfouel, Mme. Tréfouel, Nitti and Bovet (64) found not only prolongation of life but a certain percentage of recovery; the fact that these observers used rather small infective doses and strains of low mouse virulence, and in some cases infected subcutaneously and not intraperitoneally appears to explain the discrepancy. At any rate, sulfanilamide is much less active against the pneumococcus than against the hemolytic streptococcus in mice. An exception to this is the finding by Schmidt (162) of an excellent therapeutic effect in mice with type XIV pneumococcus. In rats, a more resistant animal than the mouse, a more favorable therapeutic effect of sulfanilamide against pneumococcus has been reported by Cooper and Gross (36) and Rosenthal, Bauer and Branham (158). The few studies reported for rabbits (158, 100) indicate that the drug possesses a therapeutic effect against pneumococci.

In regard to other bacterial infections against which sulfanilamide appears to exert a beneficial effect, mention must be made of its inhibitory effect on the development of experimental tuberculosis in the guinea pig (152, 21, 167, 76, 44), of its remarkable curative effect in human urinary tract infections with B. coli, Staphylococcus aureus and albus, and group B β-hemolytic streptococci (90, 95, 86, 113), and of the highly suggestive results obtained in several cases of undulant fever (77, 12). Few reports have appeared of its effect on experimental

protozoan diseases. Buttle, Gray and Stephenson (20) state that no protection is obtained with the drug against trypanosome infection in mice or malarial infection in canaries. Campbell (24) reports negative results in rabbit syphilis. On the other hand, Coggeshall (29) found that sulfanilamide had a prophylactic value and marked therapeutic effect on acute malarial infection in monkeys, but was of no value in experimental bird malaria, and Chopra and Das Gupta (25) also found a sulfanilamide derivative effective in simian malaria. In the virus diseases studied, sulfanilamide as well as "Prontosil" and "Prontosil Soluble" have in the main given completely negative results (106, 94, 118, 102). Rosenthal, Wooley and Bauer (159) report successful results with "Prontosil" but not with sulfanilamide in mice infected with the virus of lymphocytic choriomeningitis, but Ronse (154) and MacCallum and Findlay (116) failed to corroborate their findings. Dochez and Slanetz (45) found sodium sulfanilylsulfanilate effective in the treatment of dogs and ferrets infected with canine distemper, but MacIntyre and Montgomerie (117) could not confirm this result; favorable results were also claimed by Marcus and Necheles (120) for sulfanilamide and "Prontosil Soluble" in spontaneous canine distemper, but neither Plummer, Mitchell and Walker (148), working with the ferret, nor Dickerson and Whitney (43), in naturally occurring distemper in the dog, could corroborate their findings. Climenko, Crossley and Northey (28), using disulfanilamide,[2] and Oakley (144), using the glucose derivative of 4:4'-diaminodiphenylsulfone, found a significant therapeutic action of these drugs on mice infected with a moderate dosage of influenza virus. Levaditi (102, 103), Bär (3), and MacCallum and Findlay (116) found that sulfanilamide and certain other compounds have the power of protecting a certain percentage of mice from active strains of the virus of lymphogranuloma inguinale.[3] Although it is too early to assess the significance of these somewhat contradictory observations, it is quite possible that a beginning in a chemotherapeutic attack such as that on bacterial diseases has also been made on virus diseases. It is probable, however, that only certain drugs of this general group are at all effective in virus diseases.

Although the present review makes no attempt to consider the clinical

[2] This disulfanilamide is not the same compound given this name by Rosenthal, Bauer and Branham (158). For a good discussion of the question of nomenclature of the sulfonamide derivatives see Crossley, Northey and Hultquist (38).

[3] There is some question as to whether or not lymphogranuloma inguinale is a virus disease.

side of bacterial chemotherapy, it is important to realize that the brilliant results obtained with sulfanilamide and allied compounds in experimental infections in animals have been in the main duplicated in the treatment of certain infectious diseases in man. In various types of infections due to the β-hemolytic streptococcus (group A, Lancefield) dramatic cures have occurred from sulfanilamide treatment. The excellent results obtained in hemolytic streptococcus meningitis (with a mortality, under all other forms of treatment, of over 97 per cent) constitute rigid experimental proof of the effectiveness of sulfanilamide in human streptococcus infections. There is also no question of the value of the drug in meningoccus meningitis, gonococcus infections, and in urinary tract infections due to various organisms. In staphylococcus and pneumococcus infections, the evidence of the value of this type of therapy is not conclusive. Sulfanilamide has been tried in all manner of diseases, but it is too early and the cases are too few to assess at present its value in most of these.

Toxicity and pharmacological effects on specific organs and tissues. Sulfanilamide was used in human beings before much was known of its toxicity, its pharmacological or its pathological effects in animals. While it is well known that drugs may produce in human subjects toxic effects which cannot be predicted from animal experiments, the value of such animal experiments in avoiding harmful effects in human beings is too well known to need comment.

A number of determinations of the toxicity of sulfanilamide for mice have been reported by different observers. Omitting from consideration the data which are obviously inaccurate due to the use of too small a number of animals, different observers have found for the LD_{50} in grams per kilogram for oral administration 4.0 (51), 6.0 (82), 3.8 (124), 6.3 (5), 3.3 (55), 4.8 (136) and 4.2 (178). It appears that the LD_{50} can be taken as about 4.0 grams per kilogram. Halpern and Mayer (82) state that the rat is more susceptible than the mouse, but Murayama and Leake (140) found the LD_{50} to be 10.0, Molitor and Robinson, 6.2 (136), and Wien (178) 3.9 grams per kilogram. Marshall and Cutting (121) found that with the same large dose the blood concentration in the rat was much lower than in the mouse. The LD_{50} for rabbits on oral administration is given as 2.0 grams (150) and 3.0 grams per kilogram (136). Dogs appear to be more resistant (124, 82, 136) while guinea pigs seem to be even more susceptible to the drug than rabbits (82). In regard to the lower vertebrates, the chicken

(155, 27, 179) is peculiarly susceptible to sulfanilamide while the frog is very resistant and the fish quite susceptible (179).

Since sulfanilamide is not given in a single dose to treat infectious disease in man, a determination of the acute toxicity needs to be supplemented by a study of the effect of repeated doses and chronic toxicity. Little information is available in regard to this. It has been found that daily doses considerably larger than the therapeutic dose given for many weeks do not affect dogs or change the growth curve of young rats and that no pathological lesions are present when the animals are sacrificed at the end of the experiment (124, 5, 136). However, Rimington (153) found in rats given sulfanilamide in the food a marked increase in urinary and fecal porphyrin and a slight loss in weight, but no other sign of toxicity; and Hageman (81) in mice found sub-lethal doses administered for some days led to an increase in eosinophiles in the bone marrow and a deposition of hemosiderin in the spleen. Hawking (84) described in cats dying from large doses of the drug degenerative changes, i.e., chromatolysis, in the neurones of the anterior column of the spinal cord and in some of the nerve cells of the cortex and midbrain. Similar changes in the nerve cells of the brain and spinal cord of dogs receiving repeated doses of the drug have been reported (39). Contrary to all other observers, Davis, Harris and Schmeisser (41) report pathological changes in the liver, kidney, spleen, and lung from administration of the drugs to rats.

Dogs given large doses of sulfanilamide (1 to 2 grams per kilogram) exhibit salivation, vomiting, diarrhea, hyperpnea, excitement, muscular weakness, ataxia, athetotic movements, rigidity, hypesthesia, and finally coma. The symptoms in early stages of poisoning resemble those seen in dogs given large doses of ethyl alcohol; those in later stages in many respects are similar to those shown by decorticated dogs. The placing reactions, known to be due to the cerebral cortex, are absent, and the dogs appear to be blind (82, 124, 136). The symptoms shown by other mammals are in the main similar to those seen in dogs; chickens show a condition suggesting peripheral neuritis (155, 27), while the frog shows only a marked depression and paralysis which is partly, if not entirely, peripheral in nature (179).

The above symptoms suggest that in acute poisoning with sulfanilamide the central nervous system is mainly affected. When examined for its effect on smooth muscle, the frog's heart, blood pressure and respiration, it appears inert in concentrations higher than those obtained

in its therapeutic use (84, 179). The stimulation of respiration, depression of the heart, fall of blood pressure, depression of smooth muscle and rise of blood sugar, observed by Supniewski and Hano (170) are undoubtedly due to the use of the very acid hydrochloride of sulfanilamide, and are acid and not sulfanilamide effects, since they can be duplicated with an equimolecular amount of hydrochloric acid (179). A slight depression of renal function occurs with large doses of sulfanilamide, but this is only transient and of no importance (124, 136). Acidosis is produced in dogs by large doses (124).

A number of toxic reactions from sulfanilamide have been reported in patients taking the drug. The dizziness, nausea, headache, excitement, and confusion are probably of cerebral origin and similar to the cerebral symptoms seen in animals. The acidosis and cyanosis also appear to be direct toxic effects of the drug. The fever, skin reactions, hemolytic anemia, and agranulocytosis may be true idiosyncrasies to the drug (124). Although many of the minor toxic reactions are extremely unpleasant, they are not dangerous and do not necessarily contraindicate the use of the drug. Certain reactions (e.g., anemia and agranulocytosis) are extremely serious and demand discontinuance of the drug. It is interesting to recall Ehrlich's remark at the 1913 London Congress of Medicine that one must be prepared to run certain risks in order to do effective chemotherapy.

Absorption, excretion and distribution. In dogs, sulfanilamide is readily absorbed; a study of blood concentration-time curves after oral ingestion of capsules or tablets of the drug in moderate dosage (0.05 to 0.20 gram per kgm.) as well as direct determinations of absorption in sacrifice experiments indicate that the drug is nearly completely absorbed in 3 to 4 hours (126). When given in solution by mouth, absorption is much more rapid, apparently due to the fact that absorption is negligible from the stomach but extremely rapid from the intestine (122). In the mouse and rat, absorption of the drug when given by mouth is also very rapid (121, 55). In man absorption appears to be similar to that in the dog (126).

In the dog, sulfanilamide is excreted in unchanged form in the urine (126), while in other mammals which have been examined it is excreted partly in the free form and partly as a conjugated compound (66, 126). A conjugated form is excreted by the fish, chicken, mouse, rat, guinea pig, rabbit, cat, pig, cow, horse and monkey (179). This conjugated compound has been shown to be acetylsulfanilamide (the para-acetylamino derivative of benzenesulfonamide) in the human and the rab-

bit (123). Acetylsulfanilamide is practically inactive against streptococcus infections in mice. The acetylation apparently takes place in the liver (83). Fuller (66) found in the mouse an excretion in the free and conjugated form of only 65 per cent after a single dose of sulfanilamide; he believed that the remaining 35 per cent is destroyed in the body. Recently, it has been found that about 90 per cent of injected sulfanilamide can be found in the urine of the mouse in 72 hours, and 1 or 2 per cent accounted for in the tissues (179). Marshall, Emerson and Cutting (126) found 78 to 95 per cent of a dose of the drug to be excreted in unchanged form in the urine in 48 hours in the dog, and that in human beings ingesting the drug in constant dosage per day sulfanilamide balance was frequently attained. Scudi and Ratish (165), in three humans, found after a single dose recoveries of only 51, 53 and 58 per cent, but Stewart, Rourke and Allen (169) in nine patients being given the drug over several days found an average total recovery of 93.2 per cent. The question of whether all ingested sulfanilamide can be accounted for by excretion of the free and acetylated form in the urine or whether it is excreted partly in some other form or destroyed in the organism is an important one. The evidence given above is somewhat conflicting, but it appears that only a small amount is usually unaccounted for.

The excretion of sulfanilamide by the kidney in dog and man is similar to that of urea, but reabsorption by the tubules occurs to a greater extent. The clearance of the drug is definitely increased by an increased rate of urine flow (127). Excretion of the drug is very rapid in the mouse (121); a mouse requires the ingestion, on the basis of body weight, of over ten times as much sulfanilamide per day as a man to maintain the same blood concentration (179).

Sulfanilamide resembles urea and ethyl alcohol in its equal distribution in the organism; it apparently diffuses readily to all tissues and fluids of the body. In the dog, it has been shown that, with the exception of bone and fat, the various organs contain about the same amount of sulfanilamide as blood (125). It is probably present in equal concentration in all parts of the body if concentrations are expressed per unit of water, and can be used like urea to measure the water content of the organism (147). It is present in saliva, pancreatic juice, bile, exudates and transudates in a concentration slightly lower than that in blood, and readily passes into the cerebrospinal fluid (126, 125). As might be expected, it passes from the maternal to the fetal circulation in pregnant animals and humans and is present in the same con-

centration in the fetal and maternal bloods (101, 4, 168). It is curious, in view of the above findings, to note that the blood plasma contains less sulfanilamide than the corpuscles; one would expect just the reverse on the basis of water content. This rapid diffusion and penetration of the drug to all parts of the organism is undoubtedly one of the factors which make the substance a successful chemotherapeutic agent. It is important in dealing with sulfanilamide substitutes to test their diffusion and penetration in the organism.

Relation of chemical constitution to chemotherapeutic action. Two lines of research which are being pursued actively at present have for their objectives 1, to obtain more effective and less toxic drugs to use in place of sulfanilamide, and 2, to obtain drugs which are effective in bacterial infections in which sulfanilamide fails. Since many hundreds of compounds have already been tested, we can study to a limited extent the important problem of the relation of chemical constitution to effective chemotherapeutic action. In general only qualitative conclusions can be drawn, because few comparisons of the effectiveness of different compounds have been made in a really quantitative manner; the number of mice used is frequently too small and, due to differences in absorption and excretion, the doses used do not express the correct relation between the compounds. A comparison of compounds on the basis of the blood concentration necessary for a given therapeutic effect would be much more valuable. Another difficulty is the lack of a standard method for assaying the therapeutic value of new compounds. The strain of organism, the virulence and number of organisms used for infection, the amount and spacing of the dosage, the time after infection of administering the first dose, the length of the period of therapy, and the length of time the mice are observed unquestionably affect the quantitative comparison of two active compounds having different ratios of absorption and excretion. The two general methods in use for assaying a new compound are to compare the compound with sulfanilamide on the basis of average survival time of the treated mice or to compare the mortality rates of groups of mice treated with the compound and with sulfanilamide; these two methods probably do not give the same result with all compounds.

So far, all of these newer compounds which have been shown to be active against bacterial infections contain sulfur.[4] It has been demon-

[4] Buttle and collaborators (19) report slight activity with 4,4′-dinitrodiphenylmethane, 4,4′-dinitrodiphenyloxide and 2,2′dinitro-4-4′-diaminodiphenylmethane. It is also to be noted that the quinine derivatives used by Johnston

strated, however, that the sulfonamide group is not necessary for activity. Many compounds which do not contain this group and cannot yield it in the body are highly effective as bacterial chemotherapeutic agents. Thus mercaptans (71), disulfides (64), sulfinic acids (70), sulfonic acids (20), monosulfides, sulfoxides, and sulfones (63, 64, 23) containing an aromatic nucleus have all been reported to have definite therapeutic activity against streptococcus infection in mice. With few exceptions, all of these compounds which are active contain a nitro, amino, or substituted amino group in the para-position to the sulfur.[5]

Considerable information is available concerning the effect of changes in the sulfanilamide molecule on antistreptococcus activity (20, 172, 75, 135, 7, 38). Little or no activity is found in mononuclear compounds in which either the amino group is replaced by some other group or the sulfonamide group by groups not yielding a sulfonic acid on oxidation. An apparent exception to this is the activity of compounds where the amino group is replaced by a nitro group or azo linkage. This is to be explained by the ready change of such compounds to an amino compound in the organism. A shift of the amino group to the ortho or meta position results in loss of activity; also, a third group in the benzene ring results in loss or lowering of activity. Substitution of the amino group by alkyl, aralkyl, substituted alkyl or aryl, acyl, and alkylidene groups in general results in lowering or loss of activity. However, substitution on the amide nitrogen has a variable effect. Methyl and ethyl groups have little effect; higher alkyl groups decrease the activity, and p-amino- or p-nitrophenyl groups are stated to increase it. Compounds of the type of sulfanilylsulfanilamide (or polycylic chains of the same general type) have been found active; while with substituents in the parent ring the highest activity is found in the para-derivative, carboxy or sulfonic acid groups in the ortho position in the second ring give the greatest activity. Derivatives of the true disulfanilamide type have been stated recently to be active.

The question of whether or not a real specificity for different organisms exists cannot be answered dogmatically at present. The difference in activity of various compounds against organisms like streptococci, pneumococci and gonococci may be a quantitative rather than a qualitative

---

et al. (92), the anilquinoline compounds of Browning *et al.* (15), and the gold compounds of Feldt (56) and Collier (35) contain no sulfur. It is unlikely that sulfur is a necessary element in a bacterial chemotherapeutic agent.

[5] However, diacetyldihydroxdiphenylsulfone is also stated to be active (104).

difference. The fact that diaminodiphenylsulfone (23) and 2-sulfanilylaminopyridine (176) are both effective against streptococcus infections, but infinitely more effective against pneumococcus than sulfanilamide would argue for some sort of specificity, as would the limited data available on the treatment of experimental virus infections.

Mechanism of action. The intelligent use of any drug demands that its mechanism of action shall, if possible, be understood, and the logical method of attacking the problem of sulfanilamide substitutes for use in place of sulfanilamide or in infections in which this drug fails would appear to be the discovery of how sulfanilamide acts. When one realizes that in the chemotherapy of no protozoan disease is there as yet any satisfactory and generally accepted explanation of the mode of action of the drug, it is not surprising that in the short time that intensive work on the sulfonamide derivatives has been pursued no satisfactory explanation of the mechanism of action has been found. However, there is reason to believe that in many ways the attack upon the mechanism of action may be more fruitful of results in the case of bacterial than in that of protozoan chemotherapy.

Obviously, in a consideration of this problem, three factors are involved, the drug, the infecting organism, and the host. The first step in any explanation of the chemotherapeutic action is to decide to what extent the reaction between drug and bacteria is sufficient and to what extent, if any, the tissues of the host play a rôle either in changing the drug or in removing the organisms by the so-called defense mechanism. A study of the antibacterial action of the drug *in vitro* under different conditions is a necessary preliminary to studies in the infected animal.

For the original "Prontosil" and "Prontosil Soluble," all observers agree that no effect is exerted even by high concentrations of these drugs upon the streptococcus *in vitro* (48, 106, 34, 114). Indeed, Domagk's original discovery of the effect of "Prontosil" was due to the idea that compounds which were not bactericidal *in vitro* might be so *in vivo* and vice versa. With sulfanilamide, however, the situation is quite different, as most observers are convinced that bacteriostatic or bactericidal effects can be demonstrated *in vitro* with concentrations of the drug of the order of those occurring in the blood and tissues of patients undergoing treatment. Different workers have reported, as the result of their *in vitro* experiments on streptococci with concentrations of sulfanilamide which can be attained in the blood of patients,

no effect (157, 16, 59, 49), bacteriostatic (33, 114, 68, 129, 146, 50) or bactericidal effects (33, 143, 129, 58, 88, 146, 177).

The marked discrepancies shown by these *in vitro* studies are due in the main to differences in the conditions of the experiments. The strain of streptococcus, the size of the initial inoculum, the composition of the media, and the temperature are now known to have a definite effect on the result obtained in *in vitro* tests with sulfanilamide. Bliss and Long (8) state that strains of streptococci belonging to group A (Lancefield) are more susceptible to the *in vitro* action of sulfanilamide than those belonging to other groups, group D strains being highly resistant; Hoare (88) found two resistant types in the twenty-one types—all of group A which were tested. Colebrook, Buttle and O'Meara (33) state that in broth at 37° a concentration of 10 mgm. per cent sulfanilamide inhibited the growth of a small inoculum, but that 1000 mgm. per cent did not affect the growth of an inoculum of 300,000,000 organisms, but did have an effect on one-tenth this number. This dependence of the effect of the drug upon the initial concentration of bacteria has been noted by many workers, and probably explains the lack of any bacteriostatic or bactericidal effect noted by certain investigators. The composition of the media used to make the test can be seen to be a very important factor from the following observations. Bactericidal effects have been reported by Nitti, Bovet and Depierre (143) with very small inocula in Martin peptone broth with 10 mgm. per cent; by Mayer (129) in salt solution with 20 mgm. per cent; by Osgood (146) in bone marrow culture media with 10 mgm. per cent; by Colebrook, Buttle and O'Meara (33) in human and monkey blood and serum with 6 to 10 mgm. per cent but not in rabbit, guinea-pig or mouse blood. These results were thought to be mainly independent of the leucocytes, and were confirmed by Finklestone-Sayliss, Paine and Patrick (58) and Hoare (88), who state that leucocytes are not necessary for the effect. Bliss and Long (9) could not demonstrate any bactericidal effect of sulfanilamide in a concentration of 100 mgm. per cent in broth or serum broth when kept 48 hours at a low temperature (±4°C.), in the same concentration with large numbers of bacteria suspended in Tyrode's solution in 20 hours at 37°C., or in serum from patients receiving sulfanilamide (containing 5 to 10 mgm. per cent of the drug). Bürgers (17) also states, without giving any details, that the bactericidal action of horse, rabbit, guinea-pig or human serum is not increased by the addition of sulfanilamide. Colebrook (32) has

stated that to obtain the bactericidal effect in blood limited aeration is necessary; under conditions permitting free aeration, a different result is obtained. Buttle (18) and Fleming (60, 61) in their latest reports state that the leucocytes are necessary for any marked bactericidal as opposed to bacteriostatic effect in blood. The addition of a drop of blood to a tube of broth or of a drop of broth to a tube of blood decreases the antibacterial effect of sulfanilamide. Mellon and Bambas (130) found that if a culture is diluted with sodium chloride instead of broth before inoculation, a concentration of sulfanilamide may now be bactericidal whereas with broth dilutions it had no effect.

The recent work of Lockwood (111) may explain some of the discrepancies encountered in working with whole blood and serum. This investigator found that whole blood containing 10 mgm. per cent of sulfanilamide and 0.25 per cent of peptone is bactericidal towards streptococci, but that deleucocytized blood and serum under the same conditions are only bacteriostatic and not bactericidal. However, when peptone is excluded sulfanilamide in serum is bactericidal. This author suggests that sulfanilamide prevents the specialized metabolic activity required of invasive organisms by preventing the utilization of a native protein substrate.

All of the above experiments were presumably carried out at ordinary incubator temperature (37°) and probably no careful regulation was attempted. White and Parker (177) have made important observations concerning the effect of temperature upon the action of sulfanilamide *in vitro.* At 40°, a marked bactericidal effect can be obtained in broth, with concentrations of 10 and 20 mgm. per cent of sulfanilamide, while only a bacteriostatic effect is evident under the same conditions at 37°. In view of this finding, it is reasonable to suppose that some of the discrepancies noted in *in vitro* work may be due to variation in the temperature in which individual observers have worked.

An important fact about the action of sulfanilamide *in vitro* is the latent period of 2 to 4 hours before any effect is noticed even if marked bacteriostasis or sterility results in 24 hours. This is shown in experiments carried out in whole blood, deleucocytized blood and in various broth media.

A survey of the literature reviewed above on the effect of sulfanilamide on the streptococcus *in vitro* impresses one with the fact that concentrations of the order attained in the blood and tissues during treatment are definitely bacteriostatic and bactericidal under certain conditions. It is difficult to believe that some such effect does not operate

in the infected animal or man during treatment. To conclude, however, that the whole action is a "parisitotropic" effect on the bacteria in accordance with the simple conception of Ehrlich would be completely unjustified at present. No one familiar with the many exceptions and discrepancies in Ehrlich's theory, particularly as worked out for protozoan chemotherapy (40) could, with the facts available at present, be content with such a simple hypothesis of the mode of action. However, to the reviewer the conclusion appears justified, in the light of present knowledge, that the first effect in the cure of an infected animal or man is a direct action of the drug on the parasite rather than a stimulation of defense mechanisms of the host. Such defense mechanisms may, however, operate as the final stage in the chemotherapeutic process.

Before reviewing the evidence for participation of the host, it is necessary to mention certain results that have been obtained with sulfanilamide in infections of the urinary tract with bacteria other than streptococci. Here, all observers agree that sulfanilamide in such concentrations as occur in urine after its administration has a definite bacteriostatic or bactericidal action on B. coli and certain other bacteria (87, 95, 113). It is probable that in these cases the action of sulfanilamide is a direct antiseptic one on the organism, not necessitating the participation of the host, but it is only fair to state that Kenny *et al.* (95) claim some strains of B. coli to be resistant *in vitro* and not *in vivo.* With the marked effect of slight changes in media and temperature in *in vitro* studies, further experiments are necessary along this line before final conclusions can be drawn as to the mechanism of action of sulfanilamide as a urinary antiseptic.

Domagk (48) in his first paper showed that "Prontosil" therapy brought about phagocytosis of the hemolytic streptococci in infected mice; Levaditi and Vaisman (105, 107, 108) confirmed this and stated it was due to injury or destruction of the capsules of the organism by the drug. Neither Colebrook and Kenny (34) nor Long and Bliss (114) could confirm this observation as regards the capsular changes. In mice infected intraperitoneally or in rabbits intrapleurally with hemolytic streptococci, there is a decrease in the number of organisms and increase in phagocytosis in the treated animals compared to the controls, but no change in the virulence of the drug-treated organisms (9, 68, 67, 132), although morphological changes, which persist only as long as the organisms are in contact with sulfanilamide, have been described (68, 111). These observations are taken as indicating an *in vivo* bac-

teriostasis by the drug sufficient to allow the phagocytes to eradicate the infection.

At present, it seems to the reviewer that the balance of evidence indicates that sulfanilamide affects the invading organism in the sense of producing a bacteriostatic or bactericidal action which in many cases is not sufficient to effect sterilization without the coöperation of the defensive reaction of the host. It is probable that under different conditions of infection and with different concentrations of drug present, the relative rôle played by these two factors varies. Be that as it may, we have as yet very little idea of how sulfanilamide acts upon bacteria; such phenomena observed *in vitro* as the latent period of several hours, the effectiveness of low concentrations of sulfanilamide on a small inoculum, but not on a large inoculum, and the marked influence of changes in medium and temperature still await explanation. Grace (74) records the extremely interesting observation that sulfanilamide in low concentrations acts as a plant hormone. Barron and Jacobs (6) and Chu and Hastings (26) found no effect of the drug on the oxygen consumption of isolated tissue or bacteria, but their observations extended only over a period of an hour, and resting bacteria were used. It seems clear that sulfanilamide does not act like the ordinary antiseptics in destroying resting bacteria, but acts only on actively growing organisms and interferes in some way with their metabolic activities.

Mayer (129) in an attempt to explain the latent period and certain other facts in regard to the action of sulfanilamide investigated the oxidation products of this substance. He found p-hydroxylaminobenzenesulfonamide to be about 100 times as active *in vitro* as sulfanilamide and to exhibit this action without a latent period. On the basis of these observations, he assumes that this substance, formed from sulfanilamide *in vivo*, is the active agent. In work in this laboratory, which had been begun along the same line with the hydroxylamine derivative of benzenesulfonamide, we have been unable to obtain any definite evidence that the hydroxylamine derivative is the active agent, although our observations do not rule out this possibility. We have confirmed the fact that this substance is much more active than sulfanilamide *in vitro* and acts without a latent period.

Although it is not our intention in the present review to discuss the many sulfanilamide substitutes which have been described, it is obviously necessary to discuss briefly the two original compounds, "Prontosil" and "Prontosil Soluble," the introduction of which initiated the renaissance in bacterial chemotherapy. As stated before, all observers

are agreed that these compounds have no effect upon the streptococcus *in vitro*. The Tréfouels, Nitti and Bovet (173) proposed the hypothesis that the "Prontosils" had no action as such, but acted only after reduction in the body to sulfanilamide. Colebrook and Kenny (34) found that the blood and serum of patients treated with the "Prontosils" showed bacteriostatic effects against streptococci. It was also shown that strong reducing agents would reduce "Prontosil" to a substance possessing bacteriostatic properties *in vitro* (33, 114). Kellner (93) found that when "Prontosil" was given by mouth an increased color developed in the urine after diazotization and coupling with β-naphthol, which was taken to indicate the presence of sulfanilamide. However, since "Prontosil" itself gives an increased color under these conditions, the evidence is not satisfactory. Fuller (66) showed conclusively (by isolation from the urine) that a considerable amount of sulfanilamide is excreted in the urine[6] when "Prontosil" is given by mouth or "Prontosil Soluble" by injection, but did not feel that sufficient data were available to decide if the "Prontosils" acted entirely through the sulfanilamide formed. Gley and Girard (72) found a carboxy derivative of "Prontosil" to be just as active as sulfanilamide although it could yield only a third as much sulfanilamide; they used this as an argument against the theory of "Prontosil" acting through the sulfanilamide formed. Others (128, 151, 20) have found the prontosils as active as sulfanilamide, weight for weight. Feinstone, Bliss, Ott and Long (55), in a large series of mice, showed that molecule for molecule, "Prontosil Soluble" and sulfanilamide were equal in therapeutic efficiency, if given by injection immediately after infection and twice thereafter on successive days. They also found the blood levels of sulfanilamide four hours after a single dose of molecular equivalents of these two drugs to be the same, although the concentration from sulfanilamide was very much higher before this. This led them to conclude that in the mouse the action of "Prontosil Soluble" can be attributed to its breakdown to sulfanilamide.

The difficulty in interpreting all of these observations is due to the fact that where the drug is given only once a day, the blood sulfanilamide level rises sharply to a high peak and declines rapidly when sulfanilamide is given, but reaches a low level and maintains this in the blood for a longer time when "Prontosil Soluble" is given. As stated before, we do not know the relative effectiveness of these two types of blood sulf-

[6] Engel (54) has shown that liver, kidney, and blood reduce "Prontosil" to sulfanilamide *in vitro*.

anilamide levels and until this is known definite decision about the mechanism of action of the "Prontosils" cannot be made. The data available so far do not eliminate the possibility of the "Prontosils" acting entirely by slow decomposition into sulfanilamide.

In conclusion, there is no doubt that the advent of sulfanilamide and related drugs is of great significance, not only because it has altered, in a favorable sense, the prognosis of hemolytic streptococcus and other bacterial infections, but also because it has established beyond any doubt the possibility of effective chemotherapy for bacterial diseases. Moreover, from recent preliminary reports, it is quite reasonable to expect that chemotherapy may also be highly effective against certain virus diseases.

REFERENCES[7]

(1) Adolph and Lockwood. Studies in the mechanism of the action of sulfanilamide. II. Sulfanilamide in the treatment of experimental streptococcic meningitis. Arch. Otol. **27**: 535, 1938.

(2) Anselm. Unsere Erfahrungen mit Prontosil bei Puerperalfieber. Deutsch. med. Wchnschr. **61**: 264, 1935.

(3) Bär. Chemotherapeutische Versuche bei der experimentellen Infektion der Maus mit Lymphogranuloma Inguinale. Klin. Wchnschr. **17**: 588, 1938.

(4) Barker. The placental transfer of sulfanilamide. New England J. Med. **219**: 41, 1938.

(5) Barlow. Relative toxicities and therapeutic values of three chemotherapeutic agents of the sulphonamide type. Proc. Soc. Exper. Biol. and Med. **37**: 315, 1937.

(6) Barron and Jacobs. Effect of prontosil and prontylin on metabolism of bacteria. Proc. Soc. Exper. Biol. and Med. **37**: 10, 1937.

(7) Bauer and Rosenthal. Studies in chemotherapy. VII. Some new sulphur compounds active against bacterial infections. Pub. Health Repts. **53**: 40, 1938.

(8) Bliss and Long. The failure of para-aminobenzenesulfonamide therapy in urinary tract infections due to group D (Lancefield) beta hemolytic streptococci. New England J. Med. **217**: 18, 1937.

(9) Bliss and Long. Observations on the mode of action of sulfanilamide. J. A. M. A. **109**: 1524, 1937.

(10) Bliss, Long and Feinstone. The differentiation of streptococci and its relation to sulfanilamide therapy. South. Med. J. **31**: 303, 1938.

(11) Bloch-Michel, Conté, and Durel. L'emploi des dérvés sulfamidés non azöiques dans le traitement de l'erysipèle. Presse méd. **44**: 1583, 1936.

---

[7] An attempt has been made to consult all papers on the pharmacology of sulfanilamide published up to November 15, 1938. However, only those papers are quoted which bear directly on the matter discussed in the text.

(12) BLUMGART. Recovery of a patient with undulant fever treated with sulfanilamide. J. A. M. A. 111: 521, 1938.
(13) BROWN. The use of blood agar for the study of streptococci. Rockefeller Institute for Medical Research, Monograph no. 9, 1919.
(14) BROWN. Protective action of sulfanilamide and antimeningococcus serum on meningococcus infection of mice. Bull. Johns Hopkins Hosp. 61: 272, 1937.
(15) BROWNING, COHEN, ELLINGWORTH AND GULBRANSEN. The chemotherapy of experimental streptococcus infections. J. Path. and Bact. 34: 592, 1931.
(16) BÜRGERS. Chemotherapie der streptokokkenerkrankungen durch Prontosil. Deutsch. med. Wchnschr. 17: 672, 1937.
(17) BÜRGERS. Weitere Untersuchungen über den Mechanismus der Prontosilwirkung. Deutsch. med. Wchnschr. 17: 598, 1938.
(18) BUTTLE. Discussion on the treatment of bacterial diseases with substances related to sulphanilamide. Proc. Roy. Soc. Med. 31: 154, 1937.
(19) BUTTLE, DEWING, FOSTER, GRAY, SMITH AND STEPHENSON. The action of substances allied to 4:4′-diaminodiphenylsulphone in streptococcal and other infections in mice. Biochemical J. 32: 1101, 1938.
(20) BUTTLE, GRAY AND STEPHENSON. Protection of mice against streptococcal and other infections by p-aminobenzenesulphonamide and related substances. Lancet 1: 1286, 1936.
(21) BUTTLE AND PARISH. Treatment of tuberculosis in guinea pigs with sulphanilamide. Brit. M. J. 2: 776, 1938.
(22) BUTTLE, PARISH, MCLEOD AND STEPHENSON. The chemotherapy of typhoid and some other non-streptococcal infections in mice. Lancet 1: 681, 1937.
(23) BUTTLE, STEPHENSON, SMITH, DEWING AND FOSTER. The treatment of streptococcal infections in mice with 4:4′-diaminodiphenylsulphone. Lancet 1: 1331, 1937.
(24) CAMPBELL. A note on the failure of sulfanilamide to affect syphilis in the rabbit. Am. J. Syph., Gonor. and Ven. Dis. 21: 524, 1937.
(25) CHOPRA AND DAS GUPTA. A note on the therapeutic efficiency of soluseptasine in simian malaria (P. Knowlesi). Indian Med. Gaz. 73: 395, 1938.
(26) CHU AND HASTINGS. The effect of para-amino benzene sulfonamide on the oxygen consumption of tissue and certain pathogenic bacteria. J. Pharmacol. and Exper. Therap. 63: 407, 1938.
(27) CLIMENKO. Personal communication.
(28) CLIMENKO, CROSSLEY AND NORTHEY. The protective action of certain sulfanilamide derivatives in experimental influenza infections. J. A. M. A. 110: 2099, 1938.
(29) COGGESHALL. Prophylactic and therapeutic effect of sulfonamide compounds in experimental malaria. Proc. Soc. Exper. Biol. and Med. 38: 768, 1938.
(30) COHN. The effect of sulfanilamide on gonococci. An experimental study. Am. J. Syph., Gonor. and Ven. Dis. 22: 1, 1938.

(31) Cohn and Peizer. Further studies of the experimental gonococcal infection in mice and their protection by sulfanilamide. J. Infect. Dis. 63: 77, 1938.
(32) Colebrook. Discussion on the treatment of bacterial diseases with substances related to sulphanilamide. Proc. Roy. Soc. Med. 31: 149, 1937.
(33) Colebrook, Buttle and O'Meara. The mode of action of p-aminobenzenesulphonamide and prontosil in haemolytic streptococcal infections. Lancet 2: 1323, 1936.
(34) Colebrook and Kenny. Treatment of human puerperal infections, and of experimental infections in mice, with prontosil. Lancet 1: 1279, 1936.
(35) Collier. Ueber die chemotherapeutische Wirkung einer Goldverbindung eines hochmolekularen SS-Keratinspaltproduktes. Ztschr. f. Immunitätsforsch. u. exper. Therap. 85: 287, 1935.
(36) Cooper and Gross. Sulfanilamide, antipneumococcus serum and vitamin C therapy in type II pneumococcal pneumonia of rats. Proc. Soc. Exper. Biol. and Med. 36: 774, 1937.
(37) Cooper, Gross and Mellon. Action of p-aminobenzenesulfonamide on type III pneumococcus infections in mice. Proc. Soc. Exper. Biol. and Med. 36: 148, 1937.
(38) Crossley, Northey and Hultquist. Sulfanilamide derivatives. I. Aminoarysulfonamidoarylsulfonic acids and aminoarylsulfonamidoarylcarboxylic acids. J. Am. Chem. Soc. 60: 2217, 1938.
II. Disulfanilamides and related compounds. J. Am. Chem. Soc. 60: 2222, 1938.
III. Strepto-N-Polysulfanilylsulfanilamides and related compounds. J. Am. Chem. Soc. 60: 2225, 1938.
(39) Custer, Forster, LaMotte, Patton and Phinney. Changes in the central nervous system in sulfanilamide intoxication. Arch. Path. 26: 904, 1938.
(40) Dale. Chemotherapy. Physiol. Rev. 3: 359, 1923.
(41) Davis, Harris and Schmeisser. Tissue changes following prolonged administration of sulfanilamide in rats. Arch. Path. 25: 750, 1938.
(42) Dawson and Hobby. Personal communication.
(43) Dickerson and Whitney. Sulfanilamide and prontosil in the treatment of canine distemper. Proc. Soc. Exper. Biol. and Med. 38: 263, 1938.
(44) Dietrich. Prontosil in experimental tuberculosis. A preliminary report. Am. Rev. Tuberc. 38: 388, 1938.
(45) Dochez and Slanetz. The treatment of canine distemper with a chemotherapeutic agent, sodium sulfanilyl sulfanilate. Science 87: 143, 1938.
(46) Domagk. Chemotherapy of bacterial infections. Angewandte Chemie 48: 657, 1935.
(47) Domagk. Chemotherapie der Streptokokkeninfektionen. Klin. Wchnschr. 15[2]: 1585, 1936.
(48) Domagk. Ein Beitrag zur Chemotherapie der bakteriellen Infektionen. Deutsch. med. Wchnschr. 61: 250, 1935.
(49) Domagk. Untersuchungen über den Wirkungsmechanismus des Prontosil bei der experimentellen Streptokokkeninfektion. Ztschr. f. klin. Med. 132: 775, 1937.

(50) DOMAGK. Weitere Untersuchungen über den Wirkungsmechanismus des Prontosil, Uliron und seiner Derivate. Dermat. Wchnschr. 107: 60, 1938.

(51) DYKE. The relative oral toxicities for mice of the isomeric aminobenzenesulphonamides. Quart. J. Pharm. and Pharmacol. 10: 319, 1937.

(52) EHRLICH AND SHIGA. Farbentherapeutische Versuche bei Trypanosomenerkrankung. Berl. klin. Wchnschr. 41: 329, 1904.

(53) EISENBERG. Untersuchungen über halbspezifische Desinfektionsvorgänge. I. Mitteilung. Ueber die Wirkung von Farbstoffen auf Bakterien. Vitalfärbung-Entwickelungshemmung. Centralbl. f. Bakteriol. I Abt. 71: 420, 1913.

(54) ENGEL. Splitting of some diazo-derivatives in physiological medium. Acta Brevia Neerland. de Physiol. Pharmacol. Microbiol. 8: 120, 1938.

(55) FEINSTONE, BLISS, OTT AND LONG. Observations concerning the toxicity absorption and therapeutic effect of sulfanilamide and certain related organic sulphur-containing compounds in experimental infections in mice. Bull. Johns Hopkins Hosp. 62: 565, 1938.

(56) FELDT. Chemo-und Serumtherapie der Pneumokokkeninfektion. Chemotherapeutische Versuche mit Gold. Deutsch. med. Wchnschr. 61: 595, 1935.

(57) FINDLAY. Recent advances in chemotherapy. Blakiston, 1930.

(58) FINKELSTONE-SAYLISS, PAINE AND PATRICK. The bacteriostatic action of p-aminobenzenesulphonamide upon haemolytic streptococci. Lancet 2: 792, 1937.

(59) FISCHER. Beiträge zur Chemotherapie der Streptokokkeninfektion. Arch. internat. de pharmacod. et de therap. 66[2]: 131, 1937.

(60) FLEMING. The antibacterial action in vitro of 2- (p-aminobenzenesulphonamido) pyridine on penumococci and streptococci. Lancet 2: 74, 1938.

(61) FLEMING. The antibacterial power of the blood of patients receiving 2-(p-aminobenzenesulphonamido) pyridine. Lancet 2: 564, 1938.

(62) FOERSTER. Sepsis im Anschluss an ausgedehnte Periporitis. Heilung durch Streptozon. Zentralbl. f. Haut-und Geschlechts- Krankheiten 45: 549, 1933.

(63) FOURNEAU, TRÉFOUEL, MME. TRÉFOUEL, NITTI AND BOVET. Action antistreptococcique des p-amino-phénylalcoylsulfones et de quelques dérivés sulfurés voisins. Compt. rend. Soc. de biol. 127: 393, 1938.

(64) FOURNEAU, TRÉFOUEL, MME. TRÉFOUEL, NITTI AND BOVET. Rapport entre la constitution chimique et l'activité thérapeutique antimicrobienne des dérivés organiques du soufre: phénylsulfamides, diphénylsulfures, diphénylsulfones. Bull. Acad. de méd., Paris 118: 210, 1937.

(65) FUGE. Die Behandlung der Puerperalsepsis mit Prontosil. Deutsch. med. Wchnschr. 61: 1672, 1935.

(66) FULLER. Is p-aminobenzenesulphonamide the active agent in prontosil therapy? Lancet 1: 194, 1937.

(67) GAY. The method of chemotherapeutic action of sulfanilamide. Trans. Assn. Am. Physicians 53: 299, 1938.

(68) GAY AND CLARK. On the mode of action of sulfanilamide in experimental streptococcus empyema. J. Exper. Med. 66: 535, 1937.

(69) GELMO. Über Sulfamide der p-Amidobenzolsulfonsäure. J. f. praktische Chemie 77: 369, 1908.
(70) GLEY. Chimiothérapie.—Action antistreptococcique de certains dérivés sulfurés organiques. Compt. rend. Acad. d. sc. 204: 1907, 1937.
(71) GLEY AND GIRARD. Action anti-streptococcique de la thioaniline. Compt. rend. Soc. de biol. 125: 1027, 1938.
(72) GLEY AND GIRARD. Un nouveau dérivé de la sulfamido-chrysoidine trés actif contre l'infection streptococcique. Press. med. 44[2]: 1775, 1936.
(73) GMELIN. Zur Chemotherapie des Erysipels im Kindesalter. München. med. Wchnschr. 82: 221, 1935.
(74) GRACE. Note on sulphanilamide and other chemicals that act as plant growth promoting substances. Canad. J. Research. (sec. C) 16: 143, 1938.
(75) GRAY, BUTTLE AND STEPHENSON. Derivatives of p-aminobenzene-sulphonamide in the treatment of streptococcal infection in mice. Biochem. J. 31: 724, 1937.
(76) GREEY, CAMPBELL AND CULLEY. Effect of sulfanilamide on experimental tuberculosis in the guinea pig. Proc. Soc. Exper. Biol. and Med. 39: 22, 1938.
(77) GROUÈS. Sur quelques cas de mélitococcie traités par le rubiazol seul ou associé à des agents thérapeutiques spécifiques de l'affection. Lyon Med. 158: 615, 1936.
(78) GRÜTZ. Beitrag zur Kenntnis der infektiöstoxischen, Erythema-exsudativum-multiforme-ähnlichen Exantheme. Med. Klinik 30: 52, 1934.
(79) GRÜTZ. Erythema exsudativum multiforme bullosum facici colli et extremitatum. Zentralb. f. Haut- und Geschlects-Krankheiten 49: 300, 1935.
(80) HABS AND BADER. Chemotherapeutische Versuche mit "Prontosil" bei der experimentellen Streptokokkeninfektion der Meerschweinchen. Ztschr. f. Immunitätsforsch. u. exper. Therap. 94: 66, 1938.
(81) HAGEMAN. Toxicity of sulfanilamide. A study of the pathological lesions in white mice. Proc. Soc. Exper. Biol. and Med. 37: 119, 1937.
(82) HALPERN AND MAYER. Toxicité expérimentale comparée de quelques substances antistreptococciques. Press. med. 45: 747, 1937.
(83) HARRIS AND KLEIN. Acetylation of sulfanilamide by liver tissue in vitro. Proc. Soc. Exper. Biol. and Med. 38: 78, 1938.
(84) HAWKING. Pharmacological actions of sulfanilamide. Lancet 2: 1019, 1937.
(85) HEIDELBERGER AND JACOBS. Syntheses in the cinchona series. III. Azo dyes derived from hydrocupreine and hydrocupreidine. J. Am. Chem. Soc. 41: 2131, 1919.
(86) HELMHOLZ. A comparison of mandelic acid and sulfanilamide as urinary antiseptics. J. A. M. A. 109: 1039, 1937.
(87) HELMHOLZ AND OSTERBERG. Rate of excretion and bactericidal power of sulfanilamide (Prontylin) in the urine. Proc. Staff Meeting of the Mayo Clinic 12: 369, 1937.
(88) HOARE. Bactericidal changes induced in human blood and serum by sulphamido-chrysoidine and sulphanilamide. Lancet 1: 655, 1938.

(89) HÖRLEIN. Chemotherapy of infectious diseases caused by protozoa and bacteria. Proc. Roy. Soc. Med. 29: 313, 1936.

(90) HUBER. Ueber Prontosil bei der Behandlung der kindlichen Pyurie. München. med. Wchnschr. 83[2]: 2014, 1936.

(91) JACOBS. Certain aspects of the chemotherapy of protozoan and bacterial infections. Medicine 3: 165, 1924.

(92) JOHNSTON, BURCHELL, PERMAR AND MACLACHLAN. Further experiments on the effect of certain quinine derivatives on the pneumococcus. J. Pharmacol. and Exper. Therap. 61: 364, 1937.

(93) KELLNER. Etude sur l'elimination de la sulfamido-chrysoidine. Thèse pour le Doctorat en Medecine, Paris, 1936.

(94) KELSON. Use of sulfanilamide (para-amino-benzene-sulphonamide) in experimental poliomyelitis. Proc. Soc. Exper. Biol. and Med. 36: 718, 1937.

(95) KENNY, JOHNSTON, HAEBLER AND MILES. p-Aminobenzenesulphonamide in treatment of bacterium coli infections of the urinary tract. Lancet 2: 119, 1937.

(96) KINSELLA AND MUETHER. Experimental streptococcic endocarditis. Arch. Internal. Med. 62: 247, 1938.

(97) KOCH. Ueber Desinfection. Mittheilungen aus dem Kaiserlichen Gesundheitsamte 1: 234, 1881.

(98) KOLMER, RAIZISS AND RULE. Sulfanilamide and diaminodiphenylsulfone and their diacetyl derivatives in the treatment of experimental intradermal streptococcus infections of rabbits. Proc. Exper. Biol. and Med. 39: 95, 1938.

(99) KRAMER. Ueber Erfahrungen bei der Erysipelbehandlung mit Prontosil. München. med. Wchnschr. 83: 608, 1936.

(100) KREIDLER. Treatment of pneumococcal infections in rabbits with sulfanilamide. Proc. Soc. Exper. Biol. and Med. 37: 146, 1937.

(101) LEE, ANDERSON AND CHEN. Passage of sulfanilamide from mother to fetus. Proc. Soc. Exper. Biol. and Med. 38: 366, 1938.

(102) LEVADITI. L'activité thérapeutique des dérivés benzéniques sulfurés dans les maladies provoquées par les ultravirus. Compt. rend. Soc. de biol. 127: 958, 1938.

(103) LEVADITI. Chimiothérapie de la lymphogranulomatose inguinale expérimentale. Compt. rend. Soc. de biol. 128: 138, 1938.

(104) LEVADITI, GIRARD AND VAISMAN. Chimiothérapie des infections expérimentales (gonocoque, méningocoque, pneumocoque et streptocoque) par la diacétyldioxydiphénylsulfone. Compt. rend. Soc. Biol. 127: 19, 1938.

(105) LEVADITI AND VAISMAN. Action curative du chlorhydrate de 4'-sulfamido-2,4-diaminoazobenzène et de quelques dérivés similaires, dan la streptococcie expérimentale. Compt. rend. Soc. de biol. 119: 946, 1935.

(106) LEVADITI AND VAISMAN. Action curative et préventive du chlorhydrate de 4' sulfamido-2,4-diaminoazobenzène et de quelques dérivés similaires dans la streptococcie expérimentale. Press. med. 43[2]: 2097,1935.

(107) Levaditi and Vaisman. Mécanisme de l'action curative et préventive du chlorhydrate de 4'sulfamido-2.4.-diaminoazobenzene et d'aytres dérivés similaires dans l'infection streptococique expérimentale. Compt. rend. Soc. de biol. 120: 1077, 1935.
(108) Levaditi and Vaisman. Chimiothérapie.—Action curative et préventive du chlorhydrate de 4' sulfamido-2,4-diamino-azobenzéne dans l'infection streptococcique expérimentale. Compt. rend. Acad. d. sc. 200: 1694, 1935.
(109) Levaditi and Vaisman. Chimiothérapie de l'infection meningococcique expérimentale de la souris. Compt. rend. Soc. de biol. 125: 604, 1937.
(110) Levaditi and Vaisman. La toxi-infection gonococcique expérimentale et son traitement chimiothérapique. Press. med. 45²: 1371, 1937.
(111) Lockwood. Studies on the mechanism of the action of sulfanilamide. III. The effect of sulfanilamide in serum and blood on hemolytic streptococci in vitro. J. Immunology 35: 155, 1938.
(112) Long and Bliss. Observations upon the experimental and clinical use of sulphanilamide in the treatment of certain infections. Canad. M. A. J. 37: 457, 1937.
(113) Long and Bliss. Observations upon the mode of action and the clinical use of sulfanilamide in urinary tract infections. South. Med. J. 31: 308, 1938.
(114) Long and Bliss. Para-amino-benzene-sulfonamide and its derivatives. Experimental and clinical observations on their use in the treatment of β-hemolytic streptococcic infection: A preliminary report. J. A. M. A. 108: 32, 1937.
(115) Long and Bliss. The use of para amino benzene sulphonamide (sulphanilamide) or its derivatives in the treatment of infections due to beta hemolytic streptococci, pneumococci and meningococci. South. Med. J. 30: 479, 1937.
(116) MacCallum and Findlay. Chemotherapeutic experiments on the virus of lymphogranuloma inguinale in the mouse. Lancet 2: 136, 1938.
(117) MacIntyre and Montgomerie. Sodium sulphanilyl sulphanilate and canine distemper. Brit. Med. J. 1: 875, 1938.
(118) McKinley, Acree and Meek. Sulphanilamide and virus diseases. Science 87: 43, 1938.
(119) MacLachlan, Permar, Johnston and Kenney. Some effects of quinin derivatives in experimental pneumococcus studies. Am. J. Med. Sci. 188: 699, 1934.
(120) Marcus and Necheles. Treatment of spontaneous canine distemper with sulfanilamide. Proc. Soc. Exper. Biol. and Med. 38: 385, 1938.
(121) Marshall and Cutting. Absorption and excretion of sulfanilamide in the mouse and rat. Bull. Johns Hopkins Hosp. 63: 328, 1938.
(122) Marshall, Cutting and Cover. The absorption and excretion of certain sulfanilamide derivatives. Bull. Johns Hopkins Hosp. 63: 318, 1938.
(123) Marshall, Cutting and Emerson. Acetylation of para-aminobenzenesulfonamide in the animal organism. Science 85: 202, 1937.
(124) Marshall, Cutting and Emerson. The toxicity of sulfanilamide. J. A. M. A. 110: 252, 1938.

(125) MARSHALL, EMERSON AND CUTTING. The distribution of sulfanilamide in the organism. J. Pharmacol. and Exper. Therap. 61: 196, 1937.
(126) MARSHALL, EMERSON AND CUTTING. Para-aminobenzenesulfonamide. Absorption and excretion: Method of determination in urine and blood. J. A. M. A. 108: 953, 1937.
(127) MARSHALL, EMERSON AND CUTTING. The renal excretion of sulfanilamide. J. Pharmacol. and Exper. Therap. 61: 191, 1937.
(128) MAYER. Chimiothérapie expérimentale des infections streptococciques. Biol. Med. 27 (supplement): 45, 1937.
(129) MAYER. Recherches sur le mécanisme de l'action antistreptococcique des phénylamines sulfamidées. Biol. Med. 27 (supplement): 74, 1937.
(130) MELLON AND BAMBAS. New observations on the mechanism of sulfanilamide's action. The phenomenon of potentiation. Med. Rec. 146: 247, 1937.
(131) MELLON, GROSS AND COOPER. Experimental studies with sulfanilamide and with prontosil in hemolytic streptococcus infections. J. A. M. A. 108: 1858, 1937.
(132) MELLON, GROSS AND COOPER. Sulfanilamide therapy of bacterial infections with special reference to diseases caused by hemolytic streptococci, pneumococci, meningococci and gonococci. Charles C. Thomas, Baltimore, Md., 1938.
(133) MELLON, SHINN AND MCBROOM. Therapy of experimental staphylococcus infections with sulfonamide compounds. Proc. Soc. Exper. Biol. and Med. 37: 563, 1937.
(134) MEUNIER AND NITTI. L'action protectrice du p-aminobenzènesulfamide (1162 F) dans les infections streptococciques expérimentales chez le rat carencé en vitamine A. Compt. rend. Soc. de biol. 127: 688, 1938.
(135) MIETZSCH. Zur Chemotherapie der bakteriellen Infektionskrankheiten. Ber. d. deutsch. chem. Gesellsch. 71: (Heft 1, Abt. A) 15, 1938.
(137) MOLITOR AND ROBINSON. Some pharmacological and toxicological properties of sulfanilamide and benzylsulfanilamide. J. Pharmacol. and Exper. Therap. (in press).
(137) MONTESTRUC. Essais de traitement des streptococcies expérimentales du rat et du lapin per injections sous-cutanees de 1162 F, en suspension dans l'huile et dans l'huile-lanoline. Bull. Soc. de path. exot. 30: 265, 1937.
(138) MONTESTRUC. Nouveaux essais de traitement des streptococcies par injections de 1162 F en suspension huileuse. Bull. Soc. de path. exot. 30: 15, 1937.
(139) MORGENROTH AND LEVY. Chemotherapie der Pneumokokken-infektion. Berl. klin. Wchnschr. 48[2]: 1560, 1911.
(140) MURAYAMA AND LEAKE. The acute and chronic toxicity of sulfanilamide in rats. J. Pharmacol. and Exper. Therap. (Proc.) 63: 29, 1938.
(141) NITTI AND BOVET. Action du 4-sulfonamide-2-4-diaminobenzol (Prontosil) sur des infections streptococciques de la souris provoquées par des streptocoques d'origine humaine. Compt. rend. Soc. de biol. 119: 1277, 1935.

(142) NITTI AND BOVET. Chimiotherapie. Les septicémies streptocociques expérimentales et leur traitement par le p-amino-phenylsulfamide. Compt. rend. Acad. d. sc. **202**: 1221, 1936.
(143) NITTI, BOVET AND DEPIERRE. Action des dérives du p-amino-phenylsulfamide (1162 F) sur les streptocoques hemolytiques in vitro. Compt. rend. Soc. de biol. **124**: 16, 1937.
(144) OAKLEY. Chemotherapy of virus infection. Brit. Med. J. **1**: 895, 1938.
(145) OKAMOTO. Zur Chemotherapie der Pneumokokkeninfektion, insbesondere über die Wirkung des Äthylapochinins auf die Pneumokokkeninfektion der Mäuse. Japanese J. Med. Sci. IV. Pharmacology. Proc. 5th Annual Meeting **5**: 36, 1931.
(146) OSGOOD. Culture of human marrow. Studies on the mode of action of sulfanilamide. J. A. M. A. **110**: 349, 1938.
(147) PAINTER. Simultaneous determinations of the body water available for dilution of exogenous urea and sulfanilamide in unanesthetized dogs. Am. J. Physiol. **123**: 159, 1938.
(148) PLUMMER, MITCHELL AND WALKER. Experiments with sodium sulfanilyl sulfanilate. Canad. J. Comp. Med. **2**: 139, 1938.
(149) PROOM. The therapeutic action of p-aminobenzenesulphonamide in meningococcal infection of mice. Lancet **1**: 16, 1937.
(150) RAIZISS, SEVERAC AND MOETSCH. Chemotherapeutic studies of sulfamidyl (para-amino-benzene-sulfonamide) in experimental beta-hemolytic streptococcic infection. J. Chemotherapy **14**: 1, 1937.
(151) RAIZISS, SEVERAC, MOETSCH AND CLEMENCE. The chemotherapy of sulfanilamide, Prontosil Soluble, di-sulfanilamide and proseptasine. J. Chemotherapy **14**: 91, 1938.
(152) RICH AND FOLLIS. The inhibitory effect of sulfanilamide on the development of experimental tuberculosis in the guinea pig. Bull. Johns Hopkins Hosp. **62**: 77, 1938.
(153) RIMINGTON. Porphyrinuria following sulphanilamide: Sulphanilamide dermatitis. Lancet **1**: 770, 1938.
(154) RONSE. Essai de chimiothérapie dans la chorio-meningite de la souris. Compt. rend. Soc. de biol. **127**: 845, 1938.
(155) ROSENTHAL. Personal communication.
(156) ROSENTHAL. Studies in chemotherapy. II. Chemotherapy of experimental pneumococcus infections. Pub. Health Repts. **52**: 48, 1937.
(157) ROSENTHAL. Studies in chemotherapy. III. The effect of p-aminobenzenesulphonamide on pneumococci in vitro. Pub. Health Repts. **52**: 192, 1937.
(158) ROSENTHAL, BAUER AND BRANHAM. Studies in chemotherapy. IV. Comparative studies of sulphonamide compounds in experimental pneumococcus, streptococcus, and meningococcus infections. Pub. Health Repts. **52**: 662, 1937.
(159) ROSENTHAL, WOOLEY AND BAUER. Studies in chemotherapy. VI. The chemotherapy of choriomeningitis virus infection in mice with sulphonamide compounds. Pub. Health Repts. **52**: 1211, 1937.
(160) SCHAFFER. Protective effects against lethal streptococcus hemolyticus of glutamine and various sulfonamide compounds. Proc. Soc. Exper. Biol. and Med. **37**: 648, 1938.

(161) SCHERBER. Zur lokalen und allgemeinen Behandlung des Rotlauf, in letzterer Beziehung mit besonderer Darstellung der Anwendung des Farbstoffpräparates Prontosil (Streptozon, Präparat 5214) wie der Behandlungsresultate mit Omaadin. Wien. med. Wchnschr. 85: 284, 346, 376, 1935.
(162) SCHMIDT. Use of sulfanilamide in the treatment of type XIV pneumococcus infections in mice. Proc. Soc. Exper. Biol. and Med. 37: 205, 1937.
(163) SCHRANZ. Zur Sepsisbehandlung mit Prontosil. München. med. Wchnschr. 82: 419, 1935.
(164) SCHREUS. Chemotherapie des Erysipels und anderer Infektionen mit Prontosil. Deutsch. med. Wchnschr. 61: 255, 1935.
(165) SCUDI AND RATISH. The urinary excretion of single doses of sulfanilamide. J. Lab. and Clin. Med. 23: 615, 1938.
(166) SEASTONE. The effect of sulfanilamide (para-amino-benzene-sulfonamide) on groups "C" hemolytic streptococcus infections. J. Immunology 33: 403, 1937.
(167) SMITHBURN. Inefficacy of prontylin in experimental tuberculosis. Proc. Soc. Exper. Biol. and Med. 38: 574, 1938.
(168) SPEERT. The passage of sulfanilamide through the human placenta. Bull. Johns Hopkins Hospital 63: 337, 1938.
(169) STEWART, ROURKE AND ALLEN. Excretion of sulfanilamiade. J. A. M. A. 110: 1885, 1938.
(170) SUPNIEWSKI AND HANO. Über die Pharmakodynamik p-Sulfamido-aminobenzolhydrochlorids. Bull. de l'Acad. Polonaise de Sci. et de Lettres Classe de Méd. 99, 1936.
(171) TRÉFOUEL, MME. TRÉFOUEL, NITTI AND BOVET. Activité du p-aminophénylsulfamide sur les infections streptococciques expérimentales de la souris et du lapin. Compt. rend. Soc. de biol. 120: 756, 1935.
(172) TRÉFOUEL, MME. TRÉFOUEL, NITTI AND BOVET. Chimiothérapie des infections streptococciques par les dérivés du p-aminophénylsulfamide. Ann. de l'Inst. Pasteur 58: 30, 1937.
(173) TRÉFOUEL, MME. TRÉFOUEL, NITTI AND BOVET. Le mode d'action du p-aminophenylsulfamide et des dérivés azoiques dans les septicemies streptococciques expérimentales. Press. med. 45[2]: 839, 1937.
(174) VEIL. Die Therapie des Rheumatismus. Therap. der Gegenwart 75: 212, 1934.
(175) WHITBY. An experimental assessment of the therapeutic efficacy of amino compounds with special reference to p-benzylaminobenzenesulphonamide. Lancet 1: 1517, 1937.
(176) WHITBY. Chemotherapy of pneumococcal and other infections with 2-(p-aminobenzenesulphonamido) pyridine. Lancet 1: 1210, 1938.
(177) WHITE AND PARKER. The bactericidal effect of sulfanilamide upon β-hemolytic streptococci in vitro. J. Bacteriol. 36: 481, 1938.
(178) WIEN. The toxicity of 2-(p-aminobenzenesulphonamido) pyridine. Quart. J. Pharm. and Pharmacol. 11: 217, 1938.
(179). Unpublished observations from this laboratory.

THE ULTRASTRUCTURE OF PROTOPLASMIC CONSTITUENTS

FRANCIS O. SCHMITT

Department of Zoology, Washington University St. Louis, Mo.

The term ultrastructure as used in this review denotes the submicroscopic organization of cellular and tissue components and includes not only the shapes and orientations of the larger aggregates and micelles,[1] but also the configurations of the molecules which compose the micelles and the various phases of protoplasmic systems. While, strictly speaking, the determination of tissue ultrastructure is a branch of morphology, it is intimately connected with chemistry since it is essentially the morphology of molecular complexes; to learn the details of the ultrastructural organization of such systems it is necessary to know the chemical composition and the physical and chemical properties of the molecules which compose the system. It is a subject of great interest also to physiologists, for just as a knowledge of gross and microscopic anatomy was essential to the development of physiology in the past, so in the modern era with its great advances in the structural chemistry of complex biological substances, a knowledge of cellular and tissue ultrastructure must be obtained if physiologists are to make full use of the recent advances in chemistry in order to discover the mechanisms which underlie tissue and cell function. Obviously before the dynamic aspects of a system, biological or otherwise, can be studied efficiently the structure of the system must be known.

Structure determination should be regarded, therefore, as a means to an end rather than as a final goal in itself. For example, if application of optical methods succeeds in demonstrating the specific configuration of the fibrous protein molecules in resting muscle, it still remains to be determined whether this configuration is altered during

[1] The word micelle has been used rather loosely in recent years to denote structures fundamentally different from those which Nägeli had in mind when he coined the word in 1858. It may, in fact, be wise to discontinue its usage unless a satisfactory definition can be agreed upon for general adoption by chemists as well as biologists. For an illuminating account of this problem and of the micellar theory, see Frey-Wyssling (76, pp. 80–88).

contraction or tension development, and whether a general theory of contractility can be based on such facts. In view of the highly technical nature of the optical methods and the relative expense of the equipment it is desirable that the static and dynamic aspects be developed together rather than as separate investigations by morphologists and physiologists, respectively.

Until the electron microscope is developed, protoplasmic ultrastructure must be investigated by indirect methods since the particles are considerably smaller than the wave length of visible light. Data from polarization, diffraction, and interference methods must be pieced together to obtain an impression of the configuration of the entire system. And in order to interpret the optical data correctly a large body of supplementary evidence must be considered. One must be thoroughly familiar with what is known about the histology and cytology of the system being studied. For example, if one wishes to investigate a given protein structure in a tissue by x-ray diffraction one must know the relative amount and orientation of the other protein components as well. Thus if it is desired to investigate the neurofibrillar apparatus of the nerve axis cylinder by x-rays a nerve must be chosen which has a minimum of connective tissue since the latter might give rise to diffractions similar to those which might be expected from neurofibrils. It is also important that the main chemical entities composing the structure to be investigated be identified and if possible characterized. This may be difficult particularly if the structure is built of complex conjugated protein molecules. It frequently happens that data of the sort mentioned are not available in the literature and, if they appear to be of strategic importance in the interpretation of the optical data, it may become necessary to make independent investigations of these matters. Such supplementary investigations often consume more time than the original optical experiments.

Information of great value may be obtained by a consideration of the optical, physical, and chemical properties of partial systems or artificial models of biological systems. Thus valuable information has been gained about muscle ultrastructure by a consideration of the properties of artificially prepared myosin films and threads; about chromosome structure by observations on nucleic acid and nucleoprotein threads; about the myelin sheath from experiments on lipide myelin forms. Work in recent years on the structure and properties of monolayers and interfacial and built-up films has been most valuable in suggesting structures and processes which may be of importance in

biological systems. But, however suggestive such experiments on models and analogies may be, they cannot demonstrate the structure or process which is unique for the tissue or cell; this can be discovered only by investigation of the biological systems themselves by methods discussed below.

It may also be mentioned that polarized light and x-ray diffraction methods avoid certain objections frequently raised to morphological investigations since the cells and tissues may be examined in many instances in the fresh, unfixed condition. Since it can be shown that in many cases the illumination with polarized light or the brief radiation with x-rays produces no destructive effect on the system, these methods avoid the indeterminacy of the fixation methods of classical morphology, and can be relied on, when properly applied and interpreted, to reveal the structure as it exists in the living cell or tissue.

The optical techniques of use in ultrastructure investigation and their application to biological problems have until recently been most highly developed in Germany. Indeed, neither handbooks describing the use of the polarization optical analysis in biology nor detailed accounts of the results of such investigations are available in English. No attempt will be made in the present review to provide a highly condensed, hence probably unintelligible, summary of the recent German reviews and handbooks. Sufficient technical information will be given to permit the uninitiated reader to gain an idea of the scope and possibilities of the several methods and of the results which have been obtained. Present-day theories of protoplasmic ultrastructure will be reviewed in detail in the case of a few systems, chosen because they are representative and of considerable physiological interest.

I. Methodology of ultrastructure determination and interpretation of the data. A. *Direct Methods.* Ultraviolet microscopy can hardly be considered a method for the determination of ultrastructure, since the limit of resolution is of the order of 0.06–0.1μ (600–1000 AU). The method has proven useful in biology, particularly because of the fact that certain protoplasmic constituents absorb strongly in this spectral range, hence are visible without the necessity of staining (213). By a microphotometric method Caspersson (38) has made this property the basis of an extremely sensitive analytical tool by means of which the presence and location in cells of ultraviolet-absorbing substances can be determined. Since nucleic acid is such a substance the method has proven very fruitful in determining the structure and composition of chromosomes.

The most promising method of revealing the structure of submicroscopic objects directly is that of the electron microscope. Because of their charge, a beam of electrons may be deflected by a magnetic or electrostatic field. Hence in the electron microscope, electromagnetic coils or charged condensor plates are used in place of the objective and ocular lenses of the ordinary microscope. The electron rays are thus bent and focused in a manner analogous to that in an ordinary microscope and the image is produced on a photographic plate or fluorescent screen. Since the wave length of an electron beam is very small (for a 40,000 volt beam, $\lambda = 0.06$ AU), the resolution theoretically attainable is many thousands of times greater than the maximum of the ordinary microscope. Useful magnifications five to ten times that of the ordinary microscope have already been obtained and very much higher magnifications will doubtless be possible as the technique is improved.

Unfortunately the use of the method in biology is severely limited by the fact that the object to be examined must be dried, placed in a high vacuum, and subjected to an electron bombardment which is sufficient to destroy most biological objects. There is also a serious difficulty in focusing the beam on any given small region of a tissue if really high magnifications are to be sought. However, some of these difficulties will doubtless be overcome as the method is developed. Indeed, results of considerable biological interest have already been obtained (see, for example, 58, 28, 29, 91). It should be pointed out that if this direct method can be made as effective as seems theoretically possible, the indirect methods which involve polarization, interference and diffraction may be completely superseded for the analysis of objects which can be dried without too much artifact production.

A modification of an electron microscope (108) recently introduced by Scott promises to furnish useful information of an analytical sort. Instead of bombarding the tissue with an electron beam the tissue is made to emit electrons by the application of heat. Since the emission temperature is fairly critical for individual atoms such as K, Na, Ca, etc., it is possible in this way to determine the location of these substances in cells. Very clear pictures have already been obtained for the distribution of calcium in muscle and other tissues (175).

B. *Indirect Methods.* 1. *Polarization optics.* For an account of the theory and methodology of the polarization optics the reader is referred to books and articles of Schmidt (142, 151, 162), Ambronn and

Frey (6), Köhler (96), and Rinne and Berek (137). A brief definition of the terms commonly used in such work is given in recent reviews by Schmitt (168) and by Schmitt and Bear (171). It is the purpose of the ensuing section to indicate briefly the type of information which can be obtained by the method and to facilitate an understanding of subsequent sections on the use of the method for the analysis of ultrastructure.

a. Double refraction (birefringence). A tissue or cellular component is said to show double refraction if, by virtue of an anisotropic arrangement of some of its constituent molecules or submicroscopic particles, it transmits a beam of plane polarized light with different velocities depending on whether the plane of vibration of the light corresponds to certain symmetry axes of the system (such as the long and short axes of a fiber). The system has two descriptive refractive indices rather than one, hence is called birefringent. Numerically the birefringence is equal to the difference between the two descriptive refractive indices, though in practice it is determined by means of a compensator which measures the phase difference, or retardation, of the one ray behind the other in passing through the object. The compensator is adjusted so that its retardation just matches (in magnitude) and opposes (in sign) that of the object. The birefringence is obtained by dividing this retardation (usually expressed in mμ) by the thickness of the object expressed in similar units. If the refractive index for rays vibrating in a plane parallel to one direction of the object, let us say the long axis of a fiber, is greater or less than the refractive index for rays vibrating in the perpendicular direction, the object is said to show, respectively, positive or negative birefringence with reference to this direction. If the direction of the optic axis (direction along which the object shows no birefringence) is known, the sign should be that referred to this direction. In practice the sign of birefringence is determined by the characteristics of the compensator, which is calibrated with regard to sign as well as magnitude of birefringence.

Birefringence in biological objects is due to one or more of three causes. These may briefly be characterized as follows:

1. Crystalline (Eigen) birefringence is characteristic of systems in which the molecules are arranged as in a regular anisotropic array. Its magnitude is independent of the refractive index of the material surrounding the oriented molecules. In animal cells and tissues the structural components ("Bausteine") are usually of protein or lipide nature and both of these types characteristically show some degree of crystal-

linity as is evidenced by their birefringence. In general, both protein and lipide components show positive uniaxial crystalline birefringence, the optic axis indicating the orientation of the long axes of the molecules. In protein structures this means that the molecular chains extend in directions parallel with the optic axis which is also the long axis in the case of fibers. Nucleoprotein fibers are a notable exception to this rule, showing negative uniaxial crystalline birefringence instead of positive, but this difference is due to the nucleic acid component rather than to the protein component. Lipide molecules frequently have polar groups at one end of the long paraffin chains and non-polar groups at the other end. This configuration is to a large extent responsible for their tendency to orient in smectic fluid crystalline layers, the long paraffin chains being oriented perpendicular to the planes of the layers and the polar ends of the molecules in each layer being in apposition with those of the neighboring layer (37, 121, 106, 104). In protoplasmic systems lipide molecules are frequently associated with protein components and in this case the lipide molecules usually extend with long axes perpendicular to the long axis or planes of the protein chains. The signs of birefringence of the two components are therefore opposed, and this fact aids in diagnosing the chemical nature of the structure. Thus, if a fibrous structure shows crystalline birefringence which is negative with respect to the fiber axis the presence of oriented lipide may be suspected. This diagnosis may be considered as confirmed if extraction with lipide solvents abolishes the negative birefringence or reverses the sign to positive (characteristic of a protein component). The relative change in birefringence which results from lipide extraction is thus a measure of the lipide concentration in the structure. Nucleoprotein fibers also show birefringence which is negative with respect to the long axis. No confusion need occur, however, since extraction of nucleoprotein fibers not only fails to reverse their sign of birefringence or decrease its magnitude but frequently increases the negativity by desolvation.

2. *Photoelastic birefringence.* Substances, though intrinsically isotropic, may be made anisotropic by subjecting them to directional strains or compression. Such birefringence is seldom encountered in the relatively fluid protoplasmic systems, although in tissues like muscle it may play a rôle (73) and it has been suspected of being of importance in determining orientations in the developing embryo (60, but see 142). Recently it has proven useful in testing the theory (204) that nerve fibers grow out along lines of molecular orientation, the neuroblasts

being grown on a protein gel which has been subjected to mechanical forces, the orientation being detected by the photoelastic birefringence (129, 130). In such studies one must be careful to distinguish between birefringence due to mechanical orientation of anisotropic particles and that due to anisotropy produced by unidirectional mechanical forces acting on an isotropic body.

3. Form birefringence. The submicroscopic particles which compose protoplasmic systems may show birefringence merely because of certain geometrical properties regardless of whether or not the molecules within the particles are themselves oriented. If the particles are small with respect to the wave length of light, are asymmetric in shape, and are preferentially oriented the system will show "form" birefringence. Such birefringence will vanish, however, if the refractive index of the particles is equal to that of the material in which they are suspended. The greater the difference in refractive indices the greater the form birefringence.

Much information of a quantitative as well as qualitative sort may be obtained as to the shapes and relative volumes of such asymmetric particles by application of the theory of O. Wiener (205). Wiener showed that if the long axes of the asymmetric particles are parallel to the optic axis of the system the birefringence is positive; if the axes are perpendicular to the optic axis the birefringence is negative. This is of great diagnostic value, for if a tissue component shows positive form birefringence it may be concluded that the ultramicroscopic particles are rodlets with long axes parallel to the optic axis; if it shows negative form birefringence the particles are platelets with faces lying in planes perpendicular to the optic axis. The Wiener expressions are as follows:

$$\text{Rodlet form birefringence} \propto n_e^2 - n_0^2 = \frac{\delta_1\delta_2(n_1^2 - n_2^2)^2}{(\delta_1 + 1)n_2^2 + \delta_2 n_1^2}$$

$$\text{Platelet form birefringence} \propto n_e^2 - n_0^2 = -\frac{\delta_1\delta_2(n_1^2 - n_2^2)^2}{\delta_1 n_1^2 + \delta_2 n_2^2}$$

Where n_e and n_o are the refractive indices of the system for the extraordinary and ordinary rays, respectively, n_1 and n_2 are the refractive indices of the particles and the surrounding medium, respectively, δ_1 and δ_2 are the partial volumes of the particles and the surrounding medium, respectively.

Since form birefringence vanishes when $n_1 = n_2$ but crystalline bire-

fringence is independent of n_2, the relative importance of form and crystalline birefringence is determined by a technique introduced by Ambronn (1–5), namely, by immersing the tissue consecutively in fluids of different refractive indices, measuring the birefringence after each fluid has had time to penetrate. Plotting birefringence as ordinates against n_2 as abscissae one may obtain curves such as those shown in figure 1. If the birefringence is due exclusively to the form factor it will be abolished when $n_1 = n_2$ (curve B). In most biological systems both form and crystalline birefringence are present (curves A and C). In this case the magnitude of the crystalline birefringence is given by the ordinate of the minimum of the curve. In curve A we have posi-

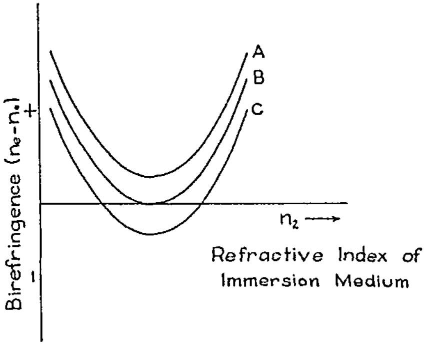


Fig. 1

tive (rodlet) form birefringence and positive crystalline birefringence. In curve C we have positive form birefringence and negative crystalline birefringence. Similarly, we may have negative (platelet) form and negative crystalline birefringence or negative form- and positive crystalline birefringence.

The Wiener relation is also of assistance in determining the relative volume, δ_1, occupied by the oriented particles. In some instances, such as in the case of muscle, sufficient is known about the protein which forms the particles to permit calculation from the form birefringence data and the Wiener relation of the volume occupied by the particles and of the changes in orientation and volume which occur during

physiological activity (see p. 288). In other cases little may be known of the properties or concentration of the substance responsible for the effects. Even in these cases, however, useful information may be obtained. Thus the steepness of the curve of form birefringence depends largely upon the product $\delta_1 \delta_2$ in the Wiener equation. The slope will be maximum when $\delta_1 = \delta_2 = 0.5$. By suitable choice of conditions and of immersion media, δ_2 can be varied within rather wide limits. From the shapes of the curves an estimate can be made of δ_1 under these experimental conditions, and by extrapolation, the value of δ_1 under conditions obtaining normally in the cell or tissue can be determined. A number of biological systems including muscle (188, 118, 202, 203, 125, 73, 74), elastoidin (72), nerve axis cylinder (18), nerve myelin sheath (82), and fibrin (59) have been investigated along somewhat similar lines.

Other useful facts which can be obtained from the form birefringence analysis are the refractive index of the particles (n_1) and the crystalline birefringence of the particles themselves (as distinguished from the crystalline birefringence of the system as a whole).

Biological systems are seldom as simple as that postulated in the ideal two-component mixed body theory of Wiener. However, even when applied to obviously multi-component systems, the analysis leads to useful approximate values. Though seldom actually done, the analysis may be made more accurate by the introduction of terms representing the partial volumes and refractive indices of extra components. Actually relatively few of the possibilities inherent in the Wiener theory have thus far been realized; only the simplest cases involving the extremes of asymmetry (infinitely extended "rodlets" or "platelets" perfectly oriented) have been considered.

b. Double absorption (*dichroism*). As anisotropic structures have different refractive indices for light polarized in certain directions, so they may also possess different absorption coefficients. With plane polarized white light the color of such objects depends upon the relation between the plane of polarization and the distinguishing directions of the object. With plane polarized monochromatic light one may observe extinction in one direction without great effect in the perpendicular direction. The directions for which the maximum effects are observed will usually coincide with the directions of greatest and smallest refractive index of the object. Objects showing this effect are said to be dichroitic or pleochroitic. One Nicol prism only is used, and this merely to determine the plane of vibration of the light.

Again, like birefringence, dichroism may be due to intrinsic factors (crystalline- or Eigen dichroism) or to form factors (form dichroism). In the former the absorbing material is present between the oriented components as crystals or molecules which are themselves oriented. In the latter case the absorbing material is unoriented. By the method of dichroism it is possible to detect anisotropy, even though the oriented particles show no crystalline birefringence and though the refractive index (but not the absorption coefficient) of particles and surrounding medium are the same.

A number of protoplasmic systems show natural dichroism (163), one example being the retinal rods, where the effect is due to the presence of visual purple (153). But the chief biological application comes from making structures artificially dichroitic by impregnating the tissue or cell with substances such as gold, silver, or certain dyes, which are themselves highly dichroitic. Thus it is possible to detect orientation in objects in which the birefringence may be too weak to be detected in other ways; the dichroitic reagent acts as an optical amplifier, as it were.

For more detailed information on the theory and applications of dichroism the reader is referred to the article by Schmidt (146). The methods and equipment are relatively simple and might well be used more generally by the cytologist as well as by the investigator of ultrastructure. Particularly promising is the fact that certain vital dyes confer dichroism to the components of living cells and tissues, hence avoid the production of artifacts unavoidable with other methods.

1. X-ray diffraction. To determine the dimensions, configurations, and orientations of the molecules of which cellular structures are made x-ray diffraction methods may be used, since the wave lengths of x-rays are smaller than the dimensions of the molecules to be studied. Since very good accounts of the theory and methodology of x-ray diffraction are available in English (44, 7, 184, 32, 54, 214), we shall consider in the present section only certain details and precautions which must be observed in the application of the method to biological problems, together with a few points which should be kept in mind in interpreting the significance of the patterns.

In some tissues, such as tendon and nerve, very large spacings have been observed (100–400 AU). To detect such large spacings it is necessary to use long wave length ($K\alpha$ lines of Cu, Fe, or Cr) x-rays, place the photographic plate at considerable distance (20–40 cm.) from the specimen, use small, well-centered beads to cut out the primary beam

(large beads may prevent the detection of large spacings which would lie close to the central beam). and collimate the beam to a very fine pencil of rays (a divergent beam would mask the presence of large spacings). Instead of using the radiation directly emitted by the tube one may use a Laue beam reflected from a crystal (for copper radiation pentaerythritol gives relatively high intensities (70, 71) though calcite, rock salt, etc., are more stable to x-rays). This has the advantage of furnishing monochromatic and relatively non-divergent radiation, hence very long specimen-to-film distances may be used.

As is well known, x-rays produce injurious effects on tissues and cells when the radiation is very intense or prolonged. It is therefore necessary to demonstrate that the amount of radiation required to produce diffraction patterns from tissues is not sufficient to render the tissue appreciably abnormal. Boehm (23) found that muscle was relatively sensitive to radiation as indicated by alterations in the demarcation potentials. Nerves appear to be less sensitive since radiation sufficient to produce patterns has little effect on action potentials (169).

The distance between the planes producing diffraction effects is calculated from the Bragg relation, $d = \frac{n\lambda}{2 \sin \theta}$. This leads to correct values where the material producing the diffraction has crystal-like regularity of its space lattice but is inapplicable to diffraction in liquids. Since smectic mesomorphic systems have essentially liquid randomness in the planes of the smectic layers the molecular structure within such layers must be determined in other ways (see 134, 201). This is of particular importance in the case of lipide systems. The facts of greatest importance which can be obtained from the diffraction analysis are: 1, interplanar distances; 2, the presence and direction of preferential orientation; 3, the approximate dimensions and degree of asymmetry of the particles or micelles, whose internal lattice structure produces the diffraction (from microphotometric measurement of the widths of the equatorial and meridional diffractions, according to the method of Laue (103); 4, the intraplanar structure, e.g., some information concerning the probable structure of the molecules lying between the confines of the regularly repeated planes (from the relative intensities of the various orders, aided by the methods of Patterson (126) and Harker (83) though these often lead to no unique solution of the structure).

2. Electron diffraction. Analyses of the structure and orientation of organic molecules of biological interest by electron diffraction have been

confined thus far to straight chain molecules which give relatively simple patterns (79, 48). Though more complex substances will doubtless be investigated eventually, interpretation of the patterns of such substances will be extremely difficult. Like the electron microscope, the method suffers from the fact that the material must be thoroughly dried and placed in a vacuum. Because of the efficiency of scattering, however, the exposure time may be very short (patterns obtainable in a few seconds). Moreover, patterns may be obtained from very thin objects, such as a few layers of fatty acid molecules (123, 120, 196, 197, 185, 186). This suggests that valuable information may be obtained by electron diffraction concerning the structure of the mammalian red cell envelope since the latter has a thickness when dried of only 150–160 AU (174). Thicker objects may be investigated by the reflection method. Since, to have appreciable penetrating power, the electrons must have an energy of at least 10,000 volts and since such electrons have very short wave lengths ($\lambda = 0.12$ AU), the method will be of value chiefly in connection with the smaller structure periods. Another difficulty about the technique arises out of its sensitivity. Mere traces of foreign material on the surface of the object may leave a record on the pattern. This has caused difficulty in the analysis of pure compounds and may prove very troublesome with biological objects.

3. *Interference methods with the visible or near-visible spectrum.* Tissues such as striated muscle which show a regular and parallel arrangement of striae or planes may act like a diffraction grating for visible light. From the grating equation, $n\lambda = d \sin \theta_n$, the distance d, between the striae or planes can be calculated, λ being the wave length of the light, n, the order of the spectrum, and θ_n the angle of diffraction of the n^{th} order spectrum. It will be seen that the method cannot be used if d is less than $\frac{1}{2}\lambda$, and is therefore of little significance for ultrastructural investigations. It has been valuable, however, in following rapid changes in d, as in muscle contraction (135, 21, 124, 140, 141).

A number of optical systems which involve interference and diffraction effects have been devised and recommended for the investigation of biological ultrastructure. No attempt will be made to discuss the variety of dark field, ultropak, and combination techniques which have been evolved; much of this work is highly empirical and of little quantitative significance. The Spierer lens (183, 176, 178) was designed to provide a brighter, clearer picture than is possible with the ordinary dark field. Thiessen (195) and Seifriz (177) have observed striae in

cellulose structures, and in cytoplasm, with the Spierer dark field system, which are said to represent aggregates of micelles. It is also said that the existence of stresses and strains in protoplasm is evidenced by the appearance of striae as viewed with the Spierer lens. The structure of muscle has also been investigated by this method (99, 100, 101). Clifford and Cameron (49) state that measurements from Spierer micrographs furnish no information concerning any unit characteristic of the material under observation, for it is inconceivable that the unit would have a dimension common to so many and so diverse substances.

The most recent method depending on interference in the visible spectrum is that of Schmitt and Waugh (174) by which the thickness and chemical composition of the red cell membrane has been determined. With this method the red cells are deposited on a specially prepared slide, hemolyzed in distilled water or other solution and dried. The intensity and color of the light which is reflected when illumination is at normal incidence depends upon the thickness and refractive index of the membrane material. Since the refractive index of the red cell envelope is similar to that of built-up stearate films made according to the method of Blodgett (22) the thickness of the dried double membranes may be determined by matching them for color and intensity against standard stearate step films (the step thicknesses of which are known). The glass slides are of high refractive index and are painted on the under side to prevent reflection of light from this surface. The cells are viewed with the aid of a vertical illuminator system similar to that used in the study of metal surfaces by reflectivity. The built-up comparison film is mounted on the stage of a separate microscope also fitted with a vertical illuminator. The red cell membranes are matched against the step film with the aid of a comparison ocular, care being taken that the illuminators and objectives are themselves matched and that a single light source is used to illuminate both systems. With critical adjustment it is thus possible to determine average thicknesses with an accuracy of ± 10 AU. The method also gives information of an analytical sort, for the membranes can be leached with lipide solvents and the thickness of the protein residue determined. Knowing the thickness, hence volume, of the lipide and protein components, their weights can readily be estimated.

II. The ultrastructure of some typical protoplasmic systems. Tissue and cellular structures may be regarded as presenting a repetition in three dimensions of essentially two types of fundamental struc-

tures. These are the linear type represented by fibers and the lamellar type represented by interfacial films, membranes, sheaths, etc. Classification according to geometrical configuration rather than according to functional, anatomical or chemical relationships emphasizes the essential *similarity in ultrastructure of all the members of each category.* It will suffice for the present review, therefore, to present the evidence concerning the ultrastructure of only a few examples of each type, chosen because of their physiological significance and because they lend themselves well to investigation; the analysis of other fibrous and lamellar structures will, in general, follow closely along the same lines. More complete accounts may be found in the recent books and articles of Schmidt (162, 164, 166) and Frey-Wyssling (76).

A. Fibrous Structures. General remarks. Animal fibers may occur as large aggregates or bundles of smaller fibrils as in the case of supporting, elastic, and contractile tissues or in the form of microscopic, cellular structures such as spindle and astral fibers, chromosomes, cilia, etc. The data of greatest significance are those which reveal molecular configurations and these are obtained by the method of x-ray diffraction. Hence the most detailed information is that which has been obtained from tissues such as hair, tendon and muscle, which lend themselves readily to x-ray analysis. It is probable, however, that with certain precautions, the conclusions regarding ultrastructure reached from the analysis of these tissues may be applied to microscopic intracellular fibrils. This seems warranted in view of the close similarity between the polarization optical properties of tissue and cellular fibrils, detailed below.

Animal fibers may be considered according to two hierarchies of submicroscopic structure. The lower of these consists of the individual protein chain molecules and their relation with their neighboring chains; investigation of this aspect requires the x-ray diffraction method of analysis. The second hierarchy consists of aggregates of protein chains in the form of submicroscopic rodlets or micelles; information concerning such structures is best obtained from polarized light studies. A higher hierarchy would, of course, be the microscopically visible fibrillae themselves.

Protein fibers display mechanical, optical, electrical and magnetic anisotropies by virtue of the fact that they are constructed of polypeptide chain molecules which are oriented with long axes parallel to each other and to the long axis of the fiber. These chains are bonded to each other laterally by linkages between the terminal groups of the

side chains, and the physical and chemical properties of the fiber are determined largely by the relative stability of this lateral bonding (see 92, 20). If the protein is composed of amino acids which have long side chains with strongly polar terminal groups, the compactness, tensile strength, swelling, contractility, etc., will be very different than in the case of proteins composed of amino acids with short side chains having non-polar terminal groups. If the protein chains are closely packed and well integrated laterally by side linkages we have a typical mechanical tissue like hair. There is very little room for water in such fibers and there is practically no metabolism. If the protein contains a higher percentage of amino acids with long side chains having polar end groups, the fiber may still be fairly compact as in the case of tendon and connective tissue. In these the water content is much higher, though the metabolism is still low. More highly solvated protein fibers are those which characterize actively metabolizing, contractile systems such as muscle and intracellular fibrils. In these the configuration of the chains may be altered rapidly and reversibly thus producing contraction and extension. Highly solvated protein chains may also occur which are not integrated into definite, visible fibrils but which may readily be recruited into such systems by (enzymatic?) desolvation. Such a highly tenuous lattice is presumably the basis of the "cystoskeleton" postulated by Peters (127, 128, 122).

Since the molecular configuration and lateral bonding of the protein chains is most easily determined in compact tissues such as hair and tendon, the most detailed information has been obtained with these tissues. The data have been of great value in the interpretation of the structure of the physiologically more interesting fibers such as muscle.

Essentially two types of fibrous architecture are recognized at present, the keratinous and the collagenous types, both being composed of longitudinally oriented polypeptide chains bonded together by side-chain linkages.

The extensive work of Astbury on keratin structure has focussed attention upon the configuration of the chains in the long axis of the fiber. Under normal conditions the chains are not fully extended but are thrown into folds (α keratin), thus greatly reducing the overall length of the fiber. Under the influence of steam, α keratin may be stretched to approximately double its length, the folded chains being thus drawn out into the fully extended condition (β keratin). These changes may be made to occur reversibly. Under appropriate conditions, β keratin may be made to shorten to a length less than that of the original α

keratin (supercontracture). In this condition the chains are presumably more extensively folded than in the α condition.

Less is known about the folding of the chains in the case of collagenous fibers. Since collagen shows the phenomenon of supercontracture strikingly (98) it is assumed that the folding may become considerably greater than in the case of α keratin or perhaps even of supercontracted keratin. Astbury (1936) assumed that a meridional spacing of 2.84 AU observed by Katz (94, 95) represented the length of the amino acid residues along the chains; this would be the case if the chains are in a *cis-* rather than a *trans-* configuration as is the case for fully extended chains such as silk where the residue length is 3.5 AU. Clark and Schaad (47), confirmed by Champetier and Fauré-Fremiet (41) find that the length of the residue is 3.25 AU (from a meridional spacing three times this size), hence the chains are relatively little folded. The lateral distance between the chains (from side chain spacings) varies from about 11–17 AU depending upon the amount of water of solvation which is distributed between them.

A striking characteristic of collagen patterns, which may also occur in keratin patterns (Astbury, 9), though it has never been carefully examined in these, is the occurrence of meridional diffractions corresponding to very large periodicities (as large as 432 AU) *along the fiber axis* (46, 47, 215, 216, 55, 45). This suggests either that the fiber consists of parallel bundles of indefinitely extended polypeptide chains along which amino acid residues are regularly repeated with a master period as high as 300–400 AU, or alternatively, and perhaps more probably, that collagen is truly crystalline, being built up by a regular arrangement of very large molecules, the large spacings indicating the molecular dimensions. The latter possibility is suggested from the experiments of Wyckoff and Corey (215, 216) which show that dissolved collagen contains large particles which can be made to aggregate again to form a "tissue" giving patterns identical with those of normal tendon. Sedimentation data are not yet available which would indicate the size of the particles and whether or not they are monodisperse. The occurrence of such large aggregates or master periods is of considerable biological interest, particularly since similar structures are postulated as representing gene levels in the chromosomes. It is to be hoped that the structure underlying these large spacings in organized collagen and other fibrous proteins will be the subject of further detailed investigation.

1. Muscle Fibers. a. *X-ray diffraction evidence.* Early investiga-

tions of muscle structure by x-ray diffraction (85, 86, 25, 27, 206, 207, 208) brought out the fact that muscle contains longitudinally oriented chains separated laterally by side chains, in agreement with the Meyer-Mark (111, 112) concept of fiber structure. Impressed with the similarity between the long-range elastic and contractile phenomena of muscle and keratin and between the diffraction patterns of relaxed muscle and hair, Astbury undertook an extensive investigation of the meaning of these similarities. Although these investigations are not yet completed and only preliminary descriptions have been published (8, 10, further details were kindly communicated to the author privately), Astbury has come to the general conclusion that in resting muscle the protein chains are arranged in a folded configuration similar to that in α keratin, and that in contraction the chains are thrown into superfolds similar to the condition of supercontracture in hair. Evidence supporting these conclusions is given below.

These investigations have been enormously facilitated by: 1, the demonstration that the contractile fibrils are composed almost exclusively of a single type of protein, myosin, hence that the contractile and elastic properties must be due to intra- or intermolecular transformation of this protein; 2, by a fairly comprehensive survey of the physical, chemical, and optical properties of myosin (202, 203, 181, 182, 62, 119, 109, 114, 115, 125, 35, 36, 73, 14, 152, 154, 156, 161, 190, 191, 192, 105, 110, 187).

Boehm and Weber (27) obtained a fiber pattern from myosin threads formed by forcing myosin solutions into water through capillary jets. Astbury and Dickinson (11, 12, full paper in preparation) have obtained patterns from myosin films which had been somewhat stretched to produce orientation of the chains, which are so similar to those of α keratin as to be superimposable; if the films are stretched further the chains may be fully extended, and a pattern similar to β keratin is obtained. Strips of myosin film not only show extensibility properties similar to keratin fibers but exhibit also supercontraction and long range elasticity. Supercontracted myosin films, like elastoidin (40), give no new type of pattern although in maximal supercontraction the pattern is essentially a disoriented β pattern (two rings).

Since the evidence seems conclusive that contraction is due to changes in configuration of myosin "molecules" and since Astbury has demonstrated in rough outline the molecular changes which occur when oriented myosin chains undergo shortening *in vitro*, it remains but to show that the pattern of contracted muscle is similar to that of super-

contracted myosin films. The chief difficulty in such work is in arranging so that the muscle shall be radiated only while in the contracted state. Astbury (private communication) states that this desideratum has been approached in the case of slow muscles like those of Mytilus and that the pattern is essentially similar to that of supercontracted myosin films. From this it would appear that muscle contraction is due to a rapidly reversible superfolding of myosin chains. The evidence is perhaps not as crucial as might be desired since the pattern of the supercontracted state does not differ strikingly from the normal α pattern. It is impossible, therefore, on the grounds of the diffraction evidence alone to rule out mechanisms such as that postulated by Bernal (1937), according to which shortening in striated muscle is not due to molecular contraction but to transformation of straight, parallel chains (in the *A* bands) into a reverse spiral configuration (such as is assumed to occur normally in the *I* bands), by means of local desolvation.

Since the pioneering work of Brücke (34) and of Engelmann (64, 66) an extensive literature has accumulated regarding the polarized light analysis of muscle ultrastructure. This literature has been ably reviewed recently by Schmidt (162, 166). We shall present only a brief summary, therefore, of the more important points, stressing those aspects which are of general application in the analysis of protein fibers.

The contractile unit of muscle is the myofibril, which is now considered to be composed exclusively of myosin. Like most protein fibers it shows positive uniaxial birefringence with optic axis parallel to the long axis of the fiber. In smooth muscle the fibrils are uniformly birefringent throughout their length. In striated muscle the myosin fibrils are also continuous but the configuration of the molecular chains varies periodically along the length of the fiber, thus producing regions which show fairly strong positive birefringence (chains oriented parallel with the long axis) alternating with regions which show very weak positive birefringence (chains presumably greatly folded or disposed at angles to the axis). These constitute the *A* (for anisotropic, also called *Q* in the older literature) and the *I* (named on the erroneous assumption of complete isotropy) bands, respectively. The chains are not folded or crossed throughout the *I* bands, for at the level of the *Z* "membrane," the fibrils show positive crystalline birefringence (162), hence for very short distances the chains are again parallel. There is also a periodic difference either in the concentration of the myosin or in the lateral distance between the chains since the *A* regions have a

higher refractive index than the *I* regions and react differently with stains. The refractive index of the *A* regions is moreover not uniform but decreases towards the middle of the bands (Henson's zone). Since this complex structure is fairly typical of striated muscle generally, and since striation appears to have been developed in order to increase the velocity of contraction, clues to the mechanism of contraction have been sought in terms of rearrangements of the myosin chains in the alternately straight and folded or crossed chain regions. The recent speculation of Bernal (20), previously mentioned, is a stimulating attempt in this direction.

Muscle shows both positive crystalline and positive form birefringence, as was demonstrated first by Stübel (188, 189). This means that the oriented myosin chains are associated to form elongated aggregates in which the chains are arranged in semi-crystalline regularity. The existence of such coherent particles is strongly indicated by the fact that solutions of myosin show stream birefringence (118, 119, 26). Weber (203) found that fibers made by forcing myosin into water through a capillary jet showed form birefringence closely similar to that of muscle. From these data, together with approximate figures for the volume of the myosin micelle (187, 202, 203) the dimensions of the micelles and their average separation in the muscle fiber were estimated. A similar analysis of the configurations of the micelles has recently been made by Fischer (73, 74, 75) for smooth muscle both in the resting and in the contracted states. He found that the birefringence of smooth muscle depends on the length of the muscle, increasing as the muscle is extended. This was confirmed by Bozler and Cottrell (31). Using a method which avoids the necessity of measuring the muscle thickness accurately Bozler and Cottrell found that the birefringence is proportional approximately to the square root of the length of the muscle. They suggest that as the muscle is extended more myosin molecules become oriented in monomolecular layers at surfaces within the fibers. The square root relationship has been confirmed recently by Fischer (75).

It has long been known that the birefringence of striated muscle decreases during contraction (200, 60, 61). v. Muralt (117) studied this optical negative variation in the case of isometric contraction of striated muscle, using a sensitive method of continuous recording. He observed a decrease of some 40 per cent, the curve showing two minima which could not well be correlated either with mechanical or with energy changes. He assumed the decrease was due to reduced crystalline birefringence,

as might have been expected from the Meyer-Mark theory of contraction. The significance of the optical negative variation has become even more uncertain as a result of recent experiments with smooth muscle. Fischer (73) finds that if the muscle is not greatly extended, contraction results in decrease in birefringence, agreeing with v. Muralt. With stretched muscle, however, this decrease may not occur; in fact, with greatly extended muscle, contraction produces an increase in birefringence. This was attributed to a photoelastic effect resulting from stretching. Bozler and Cottrell (31) claim that the photoelastic effect can be avoided by destroying the ganglion which is attached to the muscle; the muscle can then be greatly extended without development of tension. Under these conditions isometric contraction results in no change in birefringence. Applying the method to frog sartorius muscle, the decrease found by v. Muralt could be obtained only under special conditions of tension. Under other conditions little change could be observed. They believe that birefringence is not fundamentally associated with tension development or with activity in general but rather merely with the length of the muscle; if the muscle is allowed to shorten, then birefringence will decrease simply because the muscle becomes shorter, the birefringence varying as the square root of the muscle length. Confirmation of these experiments is highly desirable since the conclusions seem to disagree with the view which has become generally accepted, that tension development and shortening involve a contraction of myosin chains in the Meyer and Mark or Astbury sense or a rearrangement of chains in the micelle according to certain geometrical schemes, such as that of Bernal (20); in either case contraction should be associated with a decrease in birefringence.

2. Cellular Fibers. It has long been known that contractile cellular fibers such as cilia, flagellae, axopodia, filopodia and myonemes of protozoa, sperm tails, etc., are similar to myofibrils in showing positive uniaxial birefringence with the optic axis parallel to the fiber axis. Though it is impossible to obtain x-ray evidence of the molecular structure of these microscopic fibers, it is reasonable to suppose that they are composed of oriented protein chains, as in the case of muscle fibers, since the polarization optical properties are similar to those of muscle.

The optical properties of cilia have been examined extensively. Cilia show both positive crystalline and positive form birefringence (107, 81), hence contain elongated particles oriented parallel with the axis of the cilia. Considerable lipide is also present and under suitable

conditions the sign of birefringence may be reversed to negative due to lipide molecules adsorbed with their paraffin chains oriented perpendicular to the protein chains (143, 78). It seems probable that at least a portion of this lipide is so oriented under normal conditions, as may be judged from the experiments of Göthlin (81) and recent interpretations of Bear and Schmitt (16). The intracellular fibrillar continuations of cilia also show positive birefringence and striking dichroism (65, 146). Even the basal granules which unite the cilia with the intracellular fibrils show strong gold dichroism. This high degree of molecular organization of the ciliary mechanism is of importance not only for contractility but doubtless also for the neuroid regulation which causes all the cilia in a cell and in the epithelium to beat in a concerted metachronous manner.

Even through a fibril may be fairly birefringent, if it is extremely small in cross section, the phase difference or retardation will be proportionately small, hence it will be impossible to detect the birefringence unless the compensator is very sensitive and the illumination is intense. Since the sensitive Köhler rotating plate compensator, having total retardations of only one-tenth to one-thirtieth of a wave length, has come into general use in biological work, birefringence has been detected in extremely thin cellular fibrils. Among these are the spindle and astral fibers and chromosomes of dividing cells.

Spindle and astral fibrils show positive uniaxial birefringence with optic axis longitudinal. Runnström (139) observed this in a plant cell, *Fritillaria imperialis*, and claimed also to have demonstrated the presence of lipides associated with protein chains (reversal of birefringence after immersion in glycerine). Schmidt (157, 158) observed the positive birefringence of spindle and astral fibers in living sea urchin (*Psammechinus*) eggs and states that, like typical contractile fibers, they show positive crystalline and form birefringence. Similar optical properties were observed in the case of neurofibrils in fresh nerve cells (42) and axons (18, 19). Although the fibrils cannot be observed in the fresh nerve cells with the ordinary microscope, the picture in the polarizing microscope is that to be expected if fibrils existed in the configurations shown in fixed preparations and if the fibrils are positively birefringent with respect to their long axes. It would seem that if an oriented protein cytoskeleton, such as has been postulated by Peters (127, 128), actually exists in cells its presence should be detectable with the most refined polarization optical methods. Something of this sort may underlie the observations of Moore and Miller (116) on the cytoplasm of centrifuged sea urchin eggs.

Chromosomes represent a type of fiber of great interest and biological importance. Cytogenetic studies having demonstrated the existence of determiners of heredity and development in chromosomes, and having even arranged the relative positions of the various genes along the chromosomes according to linear maps, it is highly important that the molecular architecture of the chromosomes be investigated in order to furnish a physical basis for gene structure. While the electron microscope may eventually furnish the most conclusive evidence, polarized light studies have revealed significant facts and x-ray diffraction experiments on artificial models have contributed fundamental information.

Early attempts to discover birefringence in chromosomes were unsuccessful (136, 69, 67). It has long been known, however, that sperm nuclei are birefringent (63, 199, 64) and since sperm nuclei contain highly condensed chromosome material these optical properties suggested the lines along which chromosome structure was subsequently to be analyzed. The most detailed information on this subject is due to Schmidt (144, 145, 147, 148, 150, 151, 162).

The birefringence of sperm heads depends on the relative degree of solvation of the nucleoprotein material, hence varies with different species and with the stage of development. Typical long slender sperm heads show negative uniaxial crystalline birefringence with optic axes parallel to the long axes of the sperm heads. In earlier, less desolvated stages the sperm may show very weak positive birefringence. These facts are explained by Schmidt (149, 151) on the basis of experiments with models consisting of stretched nucleic acid gels or of fibers made by forcing concentrated solutions of α thymonucleic acid through capillary jets into alcohol. The alcohol-dehydrated fibers show negative uniaxial crystalline birefringence like desolvated sperm heads. Before desolvation the fibers may show weak positive form birefringence, indicating that the particles are rod shaped with long axes parallel to the axis of the fiber. Chromatin may therefore show either weak positive or relatively strong negative birefringence depending on whether it is relatively solvated or desolvated.

In the relatively transparent eggs of *Psammechinus*, Schmidt (158, 159) recently observed striking birefringence of chromosomes in living, dividing eggs. The birefringence develops during metaphase, is maximum during anaphase, decreases in telophase and vanishes in late telophase. The sign of birefringence is positive with respect to the axis of the spindle. This is explained by Schmidt as follows. In the interkinetic nucleus the chromatin micelles are highly solvated and relatively disoriented, hence show no birefringence. With the onset of

cell division the micelles become oriented with long axes parallel to the axis of the chromosomes and in metaphase and anaphase the desolvation is sufficient to produce marked rodlet form birefringence, though not sufficient to allow the (negative) crystalline birefringence of the chromatin micelles to overcome the (positive) form birefringence.

More recently, important information has come from a study of the giant banded chromosomes of insect salivary glands (198, 160, 165). In these unusual, highly expanded chromosomes, it had been demonstrated by Caspersson (38) that the nucleic acid is located in the chromatic bands, the achromatic bands being composed of protein. Schmidt finds that the chromatic bands, when desolvated in alcohol, show crystalline birefringence which is negative with respect to the long axis of the chromosome. This he interprets as due to the presence of oriented nucleic acid micelles. From Caspersson's data one might expect the achromatic bands to show positive birefringence typical of protein fibers, but this has not thus far been demonstrated.

Recently it has been found that thymonucleic acid occurs in solution as thin rodlet particles with a length-to-thickness ratio of about 300:1 and that these particles show strong negative fluxional birefringence (179). The micelles or "molecules" have a weight of $5 - 10 \times 10^5$ (180). Films of thymonucleate stretched 250 per cent give x-ray diffraction patterns typical of fibers, the most striking reflection along the fiber axis occurring at 3.3 AU. Since this corresponds fairly closely with the length of the amino acid residue in proteins in which the chains are fully extended (β condition), Astbury and Bell (13) suggest that in fibers the nucleic acid is anchored laterally to the protein side chains through terminal linkages of the nucleotide groups, the latter projecting perpendicularly to the axis of the nucleic acid micelles. This view, which is inconsistent with that suggested by Wrinch (209–212), is confirmed by the fact that the fibrous compound of nucleic acid with the protein clupein gives sharp diffraction patterns which resemble those of nucleic acid itself (Astbury, private communication). Since the protein primary valence chains of chromosomes, through their side chains, fit on automatically down the sides of the long nucleic acid micelles it seems probable that the nucleic acid may play an important rôle in the synthesis of chromatin material by establishing a pattern according to which new protein chains may be laid down. This suggestion is supported by direct observations on the disposition of nucleic acid in the chromosomes under certain genetically abnormal conditions (39). Since the different nucleotides may occur along the nucleic acid

micelle according to varying patterns, the importance of the nucleic acid component in determining specific genic patterns is obvious.

B. *Lamellar (Membranous) Structures.* The macroscopic and microscopic appearance of membranes and films suggests the shapes of their submicroscopic building-stones, the latter consisting of layers of molecules oriented in planes parallel to the surface of the membrane. Such structures may be composed of a few molecular layers only, as in the case of thin interfacial films, or they may be composed of dozens or hundreds of layers, as in the case of microscopically visible membranes or of sheaths which may be several microns in thickness. The molecular architecture is probably fundamentally similar regardless of the relative thickness. The most favorable case in which to determine the fundamental ultrastructure of such systems is the nerve myelin sheath, not only because it represents the most highly developed lipide-protein lamellar system but because it lends itself readily to examination by x-ray diffraction, thus permitting an investigation of the details of molecular structure. Because of their microscopic size most cellular membranes can be investigated only by the polarized light method. The analysis of myelin sheath structure illustrates in a striking way the advantages of the use of both methods in a complementary fashion. Moreover, the information regarding the general properties of lipide-protein lamellar systems which is obtained from this complex system is useful in interpreting the structure of thinner cellular membranes which can be studied only by polarized light methods.

1. The Nerve Myelin Sheath. The structure of the nerve axon sheath has been discussed at length in several recent reviews (167, 168, 162, 171). In the present section we shall present a brief summary of this information without attempting to include details which are chiefly of incidental or of historical significance.

Considering first the polarization optical properties of the sheath, we find that, like tubular myelin forms produced by the action of water on the sheath lipides, the sheath of a fresh fiber shows positive uniaxial birefringence with optic axes directed radially. This is a property of the lipides since extraction with alcohol abolishes this birefringence and leaves a protein residue which exhibits weak negative uniaxial birefringence with optic axes also radially directed. Immersing such alcohol-extracted fibers in solutions of varying refractive index shows that this protein birefringence is form birefringence. Since the sign of birefringence is negative with respect to the direction of the optic axis, the system may be assumed to be constructed of ultramicroscopic protein

lamellae oriented in planes parallel to the surface of the sheath (113, 155, 43). The normal sheath, therefore, presents a unified system consisting of concentric layers of protein interspersed with layers of lipide, the thickness of the layers being considerably less than the wave length of light. Since the lipides are long chain molecules which are optically positive with respect to their long axes it may be inferred that the lipide molecules are oriented with their chains perpendicular to the plane of the layers, i.e., radially in the sheath.

Because of the radial orientation of the optic axes the birefringence of the sheath cannot be measured in the customary manner since the optically effective thickness is no obvious function of fiber or sheath dimensions. To make such measurements possible an expression was derived (15) relating birefringence, optical retardation (measured with a compensator), and axis cylinder and fiber diameters. The birefringence of the sheaths of frog *A* fibers thus calculated was found to be 0.011 (170). This relatively high value indicates that the lipide molecules are well oriented in the radial direction within the layers of the sheath.

More detailed information about the molecular configuration of the sheath was obtained by x-ray diffraction studies (169, 171). The diffraction patterns of fresh nerves appear to be due exclusively to the myelin sheath and are essentially similar in all vertebrate nerves so far examined. The most prominent diffractions consist of a meridionally accentuated ring corresponding to a spacing of 4.7 AU, a very faint meriodonal reflection at about 9.4 AU, and a series of equatorial spots representing orders of a fundamental spacing of 170–180 AU, the period varying somewhat with different types of nerves.

In the light of the polarization optical analysis these patterns have been interpreted as follows. The identity period of approximately 171 AU is considered to represent the thickness (in the radial direction) of the concentric lamellae. Pure lipides, or mixtures of lipides as found in nerve, give spacings to be expected of double layers of molecules and in the average these spacings are less than half that of the nerve spacing. The unit lamella of the sheath is therefore assumed to contain two double layers of mixed lipide molecules oriented with long paraffin chains radial, bounded by layers of protein, probably one, or at the most only a few molecules, in thickness. The polar groups of each molecular layer are presumably in apposition at the boundaries forming "phases" which are alternately predominantly aqueous and predominantly hydrocarbon.

The 4.7 and 9.4 AU meridional diffractions are thought to represent

the average distance between the lipide chains and molecules, respectively, in the tangential direction, e.g., within the planes of the layers. In these planes the lipide molecules are distributed in a random fashion as in a liquid, though the packing is fairly close.

The polarized light and x-ray diffraction studies, therefore, lead to the conclusion that the myelin sheath, like tubular lipide myelin forms, is composed of cylindrical smectic fluid crystalline layers wrapped concentrically, the sheath being more complex than myelin forms by virtue of the inclusion between the lipide layers or sheets of neurokeratinogenic protein material; this bonding of the lipide molecules on a possibly well organized protein manifold contributes to the stability of the myelin sheath as compared with that of myelin forms.

Although only heavily myelinated fibers have been studied in detail by x-ray diffraction, the polarized light analysis has been extended to include small as well as large fibers, invertebrate as well as vertebrate, and the above general scheme has been found to be of general application over the whole range. Though, as in the case of the so-called unmyelinated fibers, the lipide concentration may be very low, its presence and typical orientation can usually be detected by immersing the fibers in strongly polar media (e.g., glucose, glycerine, salts, etc.) of high refractive index; this abolishes or reduces the protein form birefringence and unmasks the lipide birefringence which is of opposite sign (16, 43). This technique has in fact been used to discover and localize the axon sheath in the case of fibers which are surrounded by many layers of connective tissue which are indistinguishable histochemically from the true axon sheath (17).

As yet no changes in molecular orientation in the sheath have been demonstrated to be associated with the passage of the nerve impulse. This is perhaps not unexpected in view of the very small energy changes associated with the initial electrical phenomena (87) although the large changes in resistance recently reported (52) suggest some alteration of molecular structure either in the sheath as a whole or in some critical interface in the sheath. In any case, the ultrastructure and chemical composition of the sheath must influence physiological properties such as conduction-velocity, since comparisons over a wide range of fiber types show that incorporation in the sheath of large amounts of oriented lipide favors rapid conduction; in the absence of such oriented lipide, rapid conduction is achieved only by tremendous increase of fiber diameter (16, 171, 133).

2. The Cell and Nuclear Membranes. A large body of evidence from

investigations of permeability (89, 90, 88, 33, 132), tensions at cell and protoplasmic interfaces (84), electrical impedance of cell membranes (77, 50–53), direct chemical analysis of cell membranes (80, 68, 193, 194, 30), studies of models of membranes (56, 57), etc., indicates clearly that the plasma membrane is a thin lipide-protein film, probably only a few molecular layers in thickness. It is of considerable physiological importance to know the ultrastructure of this film, particularly with regard to whether it is to be considered a mosaic of lipide and protein constituents or whether it has a layered structure like the myelin sheath, with alternate hydrocarbon (lipide) and aqueous (protein) phases.

The ultrastructure of the plasma membrane may be investigated very conveniently in the case of the mammalian erythrocyte. Under appropriate conditions of hemolysis the hemoglobin may be removed from the cells leaving merely the limiting envelope surrounding an aqueous solution. The polarization optical properties of this envelope have been found to correspond closely with those of the axon sheath of invertebrate nerve fibers: lipide components contribute positive uniaxial crystalline birefringence with optic axes radial; protein components contribute negative uniaxial form birefringence, also with optic axes radial (172). Hence the membrane is a layered structure containing lipide molecules with long paraffin chains oriented perpendicular to the surface and with protein leaflets oriented with long axes tangential, i.e., with flat surfaces parallel to the surface of the membrane. The polarized light studies do not reveal whether, as in the myelin sheath, the lipide and protein components are arranged in separate and continuous layers or whether the structure is essentially that of a mosaic.

Considering the red cell membrane as a Wiener mixed body it is possible to calculate the ratio of lipide to protein constituents in the membrane by volume and by weight (173). The ratio of partial *volumes* thus determined from optical properties is 2.0. This is in very good agreement with the value 1.7 determined directly by the interference method described on p. 282. It has been found (174) that the total thickness of the rabbit red cell membrane is about 160 AU and that of this thickness about 100 AU are occupied by lipide molecules, leaving about 60 AU for the protein constituents, the ratio of partial volumes being approximately 1.7. The ratio of partial *weights* calculated from the Wiener relation is approximately 1.2. This is in fair agreement with data obtained from direct analysis of stromata (93, 131, 68), though such analyses are notoriously unreliable with respect to the protein figures because of the difficulty of removing completely the large quantities of hemoglobin from the stromatin suspension.

The relative difficulty encountered in leaching the lipide from the red cell membranes, particularly after the membranes had been dried is doubtless due to the restraining influence of the protein material and strongly suggests that the latter occurs as extensive, though perhaps not necessarily continuous, layers between the oriented lipide molecules. If the protein occurred as small leaflet patches distributed between the lipide molecules according to a mosaic, leaching would almost certainly take place very much more rapidly. This seems reasonable from the results of Langmuir, Schaefer and Wrinch (102), working with built-up films of stearate and protein.

Polarization optical effects similar to those described for the red cell envelope have been observed also in the cell membranes of marine eggs (138, 139) and of nerve cells (42). In fresh nerve cells the nuclear membrane shows a negative polarization cross indicative of the presence of protein lamellae; if oriented lipide is present its concentration is too low to be detected even by the sensitive optical method (42). It is possible, however, in certain other cells to demonstrate the presence of oriented lipide interspersed in the protein matrix of the nuclear membrane. It seems probable that the structure typical of cell and nuclear membranes as well as protoplasmic membranes and interfaces generally is that of a series of protein leaflets lying in planes parallel to the surface of the interface, possibly with a certain amount of interconnection between leaflets, and containing a variable quantity of lipide molecules oriented with long paraffin chains perpendicular to the surfaces of the protein leaflets.

REFERENCES

(1) Ambronn, H. Kolloid. Ztschr. 6: 222, 1910.
(2) Ambronn, H. Ztschr. wiss. Mikr. 32: 43, 1915.
(3) Ambronn, H. Kolloid. Ztschr. 18: 90, 273, 1916.
(4) Ambronn, H. Kolloid. Ztschr. 20: 173, 1917.
(5) Ambronn, H. Nachr. Ges. Wiss. Göttingen math. phys. Kl., 299, 1919.
(6) Ambronn, H. and A. Frey. Das Polarisationsmikroskop. Leipzig, 1926.
(7) Astbury, W. T. Fundamentals of fibre structure. Oxford, 1933.
(8) Astbury, W. T. Cold Spring Harbor Sym. on Quant. Biol. 2: 15, 1934.
(9) Astbury, W. T. Chem. Weekblad. 33: 778, 1936.
(10) Astbury, W. T. Spiers Memorial Lecture, Faraday Soc. London, 1937.
(11) Astbury, W. T. and S. Dickinson. Nature 135: 95, 1935.
(12) Astbury, W. T. and S. Dickinson. Nature 135: 765, 1935.
(13) Astbury, W. T. and F. O. Bell. Nature 141: 747, 1938.
(14) Bailey, K. Biochem. J. 31: 1406, 1937.
(15) Bear, R. S. and F. O. Schmitt. J. Opt. Soc. Am. 26: 206, 1936.
(16) Bear, R. S. and F. O. Schmitt. J. Cell. and Comp. Physiol. 9: 275, 1937.
(17) Bear, R. S., F. O. Schmitt and J. Z. Young. Proc. Roy. Soc. London, Ser. B 123: 496, 1937.

(18) Bear, R. S., F. O. Schmitt and J. Z. Young. Proc. Roy. Soc. London, Ser. B **123**: 505, 1937.

(19) Bear, R. S., F. O. Schmitt and J. Z. Young. Proc. Roy. Soc. London, Ser. B **123**: 520, 1937.

(20) Bernal, J. D. "A speculation on muscle" in Perspectives in biochemistry. Edited by J. Needham and D. E. Green, Cambridge, 1938.

(21) Bernstein, J. Pflüger's Arch. **61**: 285, 1895.

(22) Blodgett, K. B. J. Am. Chem. Soc. **57**: 1007, 1935.

(23) Boehm, G. Ztschr. Biol. **92**: 45, 1931.

(24) Boehm, G. Kolloid. Ztschr. **62**: 22, 1933.

(25) Boehm, G. and K. F. Schotzky. Naturwiss. **18**: 282, 1930.

(26) Boehm, G. and R. Signer. Helv. Chimica Acta **14**: 1370, 1931.

(27) Boehm, G. and H. Weber. Kolloid. Ztschr. **61**: 269, 1932.

(28) Borries, B. V., E. Ruska and H. Ruska. Wiss. Veröffentlichungen aus den Siemens-Werken. **17**: 9, 1938.

(29) Borries, B. V., E. Ruska and H. Ruska. Veröffenlichungen aus den Siemens-Werken. **17**: 107, 1938.

(30) Boyd, E. M. J. Biol. Chem. **115**: 37, 1936.

(31) Bozler, E. and C. L. Cottrell. J. Cell. Comp. Physiol. **10**: 165, 1937.

(32) Bragg, W. H. and W. L. Bragg. The crystalline state. New York, 1934.

(33) Brooks, S. C. Am Naturalist **72**: 124, 1938.

(34) Brücke, E. Deutschr. Akad. Wiss. Wien. Math-naturw. **15**: 69, 1858.

(35) Buchthal, F. J. Physiol. **90**: 37P., 1937.

(36) Buchthal, F., G. G. Knappeis and J. Lindhard. Skand. Arch. f. Physiol. **73**: 163, 1936.

(37) Bungenberg de Jong, H. G. and J. Bonner. Protoplasma **24**: 198, 1935.

(38) Caspersson, T. Skand. Arch. f. Physiol. **73**: Suppl. 8, 151, 1936.

(39) Caspersson, T. and J. Schultz. Nature **142**: 294, 1938.

(40) Champetier, G. and F. Fauré-Fremiet. J. Chim. phys. **34**: 197, 1937.

(41) Champetier, G. and F. Fauré-Fremiet. J. Chim. phys. **35**: 2[illegible]3, 1938.

(42) Chinn, P. J. Cell. Comp. Physiol. **12**: 1, 1938.

(43) Chinn, P. and F. O. Schmitt. J. Cell. and Comp. Physiol. **9**: 289, 1937.

(44) Clark, G. L. Applied x-rays. New York, 1932.

(45) Clark, G. L. Biodynamica, no. 26, 1937.

(46) Clark, G. L., E. A. Parker, J. A. Schaad and W. J. Warren. J. Am. Chem. Soc. **57**: 1509, 1935.

(47) Clark, G. L. and J. A. Schaad. Radiology **27**: 339, 1936.

(48) Clark, G. L. and E. Wolthius. J. Chem. Ed. **15**: 64, 1938.

(49) Clifford, A. T. and F. K. Cameron. Ind. Eng. Chem. **26**: 1209, 1934.

(50) Cole, K. S. Trans. Faraday Soc. **33**: 966, 1937.

(51) Cole, K. S. and H. J. Curtis. Cold Spring Harbor Symposia on Quant. Biol. **4**: 73, 1936.

(52) Cole, K. S. and H. J. Curtis. Nature **142**: 209, 1938.

(53) Cole, K. S. and H. J. Curtis. J. Gen. Physiol. **22**: 37, 1938.

(54) Compton, A. H. and S. K. Allison. X-rays in theory and experiment. New York, 1935.

(55) Corey, A. B. and R. W. G. Wyckoff. J. Biol. Chem. **114**: 407, 1936.

(56) Danielli, J. F. J. Cell. Comp. Physiol. **7**: 393, 1936.

(57) DANIELLI, J. F. AND H. DAVSON. J. Cell. Comp. Physiol. 5: 495, 1935.
(58) DRIEST, E. AND H. O. MÜLLER. Ztschr. wiss. Mikr. 52: 53, 1935.
(59) DUNGERN, M. v. Ztschr. Biol. 98: 136, 1937.
(60) EBNER, V. v. Untersuchungen uber die Ursachen der Anisotropie organisierter Substanzen. Leipzig, 1882.
(61) EBNER, V. v. Pflüger's Arch. 163: 179, 1916.
(62) EDSALL, J. T. J. Biol. Chem. 89: 289, 1930.
(63) EHRENBERG, F. Ber. Verh. preuss. Akad. *Berlin 238, 1848.*
(64) ENGELMANN, T. W. Pflüger's Arch. 11: 432, 1875.
(65) ENGELMANN, T. W. Pflüger's Arch. 23: 505, 1880.
(66) ENGELMANN, T. W. Pflüger's Arch. 25: 538, 1881.
(67) ENGELMANN, T. W. Sitzungsber. Akad. Wiss. Berlin, 694, 1906.
(68) ERICKSON, B. N., H. H. WILLIAMS, S. S. BERNSTEIN, J. AVRIN, R. L. JONES AND I. G. MACY. J. Biol. Chem. 122: 515, 1938.
(69) ERRERA. C. R. Soc. Bot. Belgique 29: 1890.
(70) FANKUCHEN, F. Nature 139: 193, 1937.
(71) FANKUCHEN, F. Phys. Rev. 53: 910, 1938.
(72) FAURÉ-FREMIET, M. E. Arch. d'Anat. Mic. 32: 249, 1936.
(73) FISCHER, E. Cold Spring Harbon Sym. on Quant. Biol. 4: 214, 1936.
(74) FISCHER, E. J. Cell. and Comp. Physiol. 8: 503, 1936.
(75) FISCHER, E. Am. J. Physiol. 123: 65, 1938.
(76) FREY-WYSSLING, A. Submikroskopische Morphologie des Protoplasmas und seiner Derivate. Protoplasma Monograph, no. 15, Berlin, 1938.
(77) FRICKE, H. Cold Spring Harbor Sym. on Quant. Biol. 1: 117, 1933.
(78) GELEI, J. v. Ztschr. f. Anat. Entwicklungsgesch. 81: 530, 1926.
(79) GERMER, L. H. J. Applied Phys. 9: 143, 1938.
(80) GORTER, E. AND F. GRENDEL. J. Exper. Med. 41: 439, 1925.
(81) GÖTHLIN, G. F. Kungl. Svensk. vetensk. Handl. 51: 1, 1913.
(82) HARDERS-STEINHÄUSER, M. Ztschr. wiss. Mikr. 54: 339, 1937.
(83) HARKER, D. J. Chem. Phys. 4: 381, 1936.
(84) HARVEY, E. N. AND J. F. DANIELLI. Biol. Rev. 13: 317, 1938.
(85) HERZOG, R. O. AND W. JANKE. Festschr. Kais. Wilh. Ges. z. Forderung. d. Wiss. z. 10 jähr. Jubiläum 118, 1921.
(86) HERZOG, R. O. AND W. JANCKE. Naturwiss. 14: 1223, 1926.
(87) HILL, A. V. Proc. Roy. Soc. B 113: 345, 1933.
(88) HÖBER, R. Physiol. Rev. 16: 52, 1936.
(89) JACOBS, M. H. Ergeb. Biol. 7: 1, 1931.
(90) JACOBS, M. H. J. Applied Phys. 9: 81, 1938.
(91) JOHNSON, R. P. J. Applied Phys. 9: 508, 1938.
(92) JORDAN-LLOYD, D. Biol. Rev. 254, 1932.
(93) JORPES, E. Biochem. J. 26: 1488, 1932.
(94) KATZ, J. R. AND J. C. DERKSEN. Rec. Trav. Chim. 51: 513, 1932.
(95) KATZ, J. R. Die Röntgenspektrographie als Untersuchungsmethode. Berlin, 1934.
(96) KÖHLER, A. Handb. biol. Arbeitsmeth., Abt. ii, Teil 2, 907, 1926.
(97) KÜNTZEL, A. *Collegium* 4: 207, 1929.
(98) KÜNTZEL, A. AND F. PRAKKE. Biochem. Ztschr. 267: 243, 1933.
(99) LANGELAAN, J. W. Arch. Ne'erl. de Physiol. 19: 445, 1934.

(100) Langelaan, J. W. Arch. Ne'erl. Physiol. **21**: 6, 1936.
(101) Langelaan, J. W. Arch. Ne'erl. Physiol. **23**: 4, 1938.
(102) Langmuir, I., V. J. Schaefer and D. Wrinch. Science **85**: 76, 1937.
(103) Laue, M. v. Ztschr. Krist. **64**: 115, 1926.
(104) Lawrence, A. S. C. J. Roy. Mic. Soc. **58**: 30, 1938.
(105) Liang, T. Y. Chinese J. Physiol. **10**: 327, 1936.
(106) Macheboeuf, M. Act. Sci. et Ind., no. 448, 1936.
(107) Mackinnon, D. L. and F. Vlès. C. R. Acad. Paris **147**: 1908.
(108) McMillen, J. H. and G. H. Scott. Rev. Sci. Inst. **8**: 121, 1937.
(109) Mehl, J. W. J. Biol. Chem. **123**: lxxxiii, 1938.
(110) Meyer, K. Biochem. Ztschr. **266**: 137, 1933.
(111) Meyer, K. H. Biochem. Ztschr. **214**: 253, 1929.
(112) Meyer, K. H. and H. Mark. Der Aufbau der hochpolymeren organischen Naturstoffe. Leipzig, 1930.
(113) Mezzino, L. Riv. Biol. **13**: 31, 1931.
(114) Mirsky, A. E. J. Gen. Physiol. **19**: 559, 571, 1936.
(115) Mirsky, A. E. J. Gen. Physiol. **20**: 455, 461, 1937.
(116) Moore, A. R. and W. A. Miller. Proc. Soc. Exper. Biol. and Med. **36**: 835, 1937.
(117) Muralt, A. v. Pflüger's Arch. **230**: 299, 1932.
(118) Muralt, A. v. and J. T. Edsall. Trans. Faraday Soc. **26**: 837, 1930.
(119) Muralt, A. v. and J. T. Edsall. J. Biol. Chem. **89**: 315, 351, 1930.
(120) Murison, C. A. Phil. Mag. **17**: 201, 1934.
(121) Nageotte, J. Act. Sci. et Industr., no. 431., Paris, 1936.
(122) Needham, J. Order and life—Terry Lectures, New Haven, 1936.
(123) Nelson, H. R. Phys. Rev. **44**: 717, 1933.
(124) Nicolai, L. Pflüger's Arch. **237**: 399, 1936.
(125) Noll, D. and H. H. Weber. Pflüger's Arch. **235**: 234, 1934.
(126) Patterson, A. L. Ztschr. f. Krist. (A) **90**: 517, 1935.
(127) Peters, R. A. J. State Med. **37**: 1, 1929.
(128) Peters, R. A. Proc. Roy. Soc. B **121**: 587, 1937.
(129) Pfeiffer, H. H. Arch. Zellforsch. **19**: 139, 1937.
(130) Pfeiffer, H. H. Naturwiss. **23**: 382, 1938.
(131) Ponder, E. The mammalian red cell and the properties of hemolytic systems. Berlin, 1934.
(132) Ponder, E. Trans. Faraday Soc. **33**: 947, 1937.
(133) Pumphrey, R. J. and J. Z. Young. J. Exper. Biol. **15**: 453, 1938.
(134) Randall, J. T. The diffraction of x-rays and electrons by amorphous solids, liquids and gases. New York, 1934.
(135) Ranvier, L. Arch. de Physiol. **6**: 775, 1874.
(136) Retzius, G. Biologische Untersuchungen II, 1881.
(137) Rinne, R. and M. Berek. Anleitung zu optischen Untersuchungen mit dem Polarisationsmikroskop. Leipzig, 1934.
(138) Runnström, J. Protoplasma **4**: 388, 1928.
(139) Runnström, J. Protoplasma **5**: 201, 1929.
(140) Sandow, A. J. Cell. and Comp. Physiol. **9**: 37, 1936.
(141) Sandow, A. J. Cell. and Comp. Physiol. **9**: 55, 1936.
(142) Schmidt, W. J. Die Bausteine des Tierkörpers in Polarisiertem Lichte. Bonn, 1924.

(143) Schmidt, W. J. Ztschr. Morph. u. Ökol. 4: 674, 1925.
(144) Schmidt, W. J. Naturwiss. 16: 900, 1928.
(145) Schmidt, W. J. Zool. Jahrb. Allg. Zool. 45: 177, 1928.
(146) Schmidt, W. J. Handb. Biol. Arbeitsmeth., Abt. V. Teil 2/2, 1835, 1931.
(147) Schmidt, W. J. Arch. Prot. 77: 463, 1932.
(148) Schmidt, W. J. Arch. Prot. 78: 613, 1932.
(149) Schmidt, W. J. Naturwiss. 20: 658, 1932.
(150) Schmidt, W. J. Handw. d. Naturwiss., II Aufl. Jena 5: 167, 1934.
(151) Schmidt, W. J. Abd. Handb. Biol. Arbeitsmeth. Abt. V, Teil 10, 435, 1934.
(152) Schmidt, W. J. Ztschr. Zellforsch. mikr. Anat. 21: 224, 1934.
(153) Schmidt, W. J. Ztschr. Zellforsch. mikr. Anat. 22: 485, 1935.
(154) Schmidt, W. J. Ztschr. Zellforsch. mikr. Anat. 23: 201, 1935.
(155) Schmidt, W. J. Ztschr. f. Zellforschung mik. Anat. 23: 657, 1936.
(156) Schmidt, W. J. Ztschr. wiss. Mikr. 52; 397, 1936.
(157) Schmidt, W. J. Ber. Oberhess. Ges. Natur- u. Heilk. 17: (Naturwiss. Abt.) 140, 1936.
(158) Schmidt, W. J. Naturwiss. 24: 463, 1936.
(159) Schmidt, W. J. Arch. exper. Zellforsch. 19: 352, 1937.
(160) Schmidt, W. J. Naturwiss. 25: 506, 1937.
(161) Schmidt, W. J. Zellforsch. mikr. Anat. 26: 733, 1937.
(162) Schmidt, W. J. Die Doppelbrechung von Karyoplasma, Zytoplasma und Metaplasma. Berlin, 1937.
(163) Schmidt, W. J. Ztschr. wiss. Mikr. 54: 379, 1937.
(164) Schmidt, W. J. Protoplasma 29: 300, 435, 1937.
(165) Schmidt, W. J. Naturwiss. 26: 413, 1938.
(166) Schmidt, W. J. Naturwiss. 26: 481, 1938.
(167) Schmitt, F. O. Cold Spring Harbor Sym. on Quant. Biol. 4: 7, 1936.
(168) Schmitt, F. O. J. Applied Phys. 9: 109, 1937.
(169) Schmitt, F. O., R. S. Bear and G. L. Clark. Radiology 25: 131, 1935. Second paper in press.
(170) Schmitt, F. O. and R. S. Bear. J. Cell. and Comp. Physiol. 9: 261, 1937.
(171) Schmitt, F. O. and R. S. Bear. Biol. Rev. 14, 27, 1939.
(172) Schmitt, F. O., R. S. Bear and E. Ponder. J. Cell. and Comp. Physiol. 9: 89, 1936.
(173) Schmitt, F. O., R. S. Bear and E. Ponder. J. Cell. and Comp. Physiol. 11: 309, 1938.
(174) Schmitt, F. O. and D. F. Waugh. Anat. Rec. 73: 1939.
(175) Scott, G. H. and D. M. Packer. Anat. Rec. In press.
(176) Seifriz, W. J. Phys. Chem. 35: 118, 1931.
(177) Seifriz, W. Protoplasma 21: 129, 1934.
(178) Seifriz, W. Ind. Eng. Chem. 28: 136, 1936.
(179) Signer, R. Trans. Faraday Soc. 32: 296, 1936.
(180) Signer, R., T. Caspersson and E. Hammarsten. Nature 141: 122, 1938.
(181) Smith, E. C. J. Soc. Chem. Ind., London 103: 351 T, 1934.
(182) Smith, E. C. Proc. Roy. Soc. (B) 124: 136, 1937.
(183) Spierer, C. Arch. Sci. Phys. Nat. (Genève) 8: 121, 1926.
(184) Sponsler, O. L. Quart. Rev. Biol. 8: 1, 1933.
(185) Storks, K. H. and L. H. Germer. J. Chem. Phys. 5: 131, 1937.
(186) Storks, K. H. J. Am. Chem. Soc. 60: 1753, 1938.

(187) Stöver, R. Biochem. Ztschr. 259: 269, 1933.
(188) Stübel, H. Pflüger's Arch. 201: 629, 1923.
(189) Stübel, H. and T. Liang. Chinese J. Physiol. 2: 139, 1928.
(190) Studnitz, G. v. Ztschr. Vergl. Physiol. 21: 440, 1934.
(191) Studnitz, G. v. Ztschr. Zellforsch. mik. Anat. 23: 1, 1935.
(192) Studnitz, G. v. Ztschr. Zellforsch. mik. Anat. 23: 270, 1935.
(193) Thannhauser, S. J. and P. Setz. J. Biol. Chem. 116: 533, 1936.
(194) Thannhauser, S. J., P. Setz and J. Benotti. J. Biol. Chem. 126: 785, 1938.
(195) Thiessen, R. Ind Eng. Chem. 24: 1032, 1932.
(196) Trillat, J. J. Kolloid. Ztschr. 69: 378, 1932.
(197) Trillat, J. J. and H. Motz. Ann. Phys. (11) 4: 273, 1935.
(198) Ullrich, H. Planta 26: 311, 1937.
(199) Valentin, G. Die Untersuchung der Pflanzen-und der Thiergewebe in polarisiertem Lichte. Leipzig, 1861.
(200) Valentin, G. Pflüger's Arch. 21: 307, 1880.
(201) Warren, B. E. Phys. Rev. 44: 969, 1933.
(202) Weber, H. H. Ergeb. Physiol. 36: 109, 1933.
(203) Weber, H. H. Pflüger's Arch. 235: 205, 1934.
(204) Weiss, P. J. Exper. Zool. 68: 393, 1934.
(205) Wiener, O. Abh. Sächs. Ges. (Akad.) Wiss. 32: 509, 1912.
(206) Worschitz, F. Fortschr. Röntgenstr. 50: 174, 1934.
(207) Worschitz, F. and J. v. Hermann. Forschr. Röntgenstr. 50: 178, 1934.
(208) Worschitz, F. Fortschr. Röntgenstr. 51: 81, 1935.
(209) Wrinch, D. M. Nature 134: 978, 1934.
(210) Wrinch, D. M. Nature 135: 788, 1935.
(211) Wrinch, D. M. Nature 136: 68, 1935.
(212) Wrinch, D. M. Protoplasma, 25: 550, 1936.
(213) Wyckoff, R. W. G. Cold Spring Harbor Sym. on Quant. Biol. 2: 39, 1934.
(214) Wyckoff, R. W. G. Suppl. 1935. The structure of crystals. New York, 1931.
(215) Wyckoff, R. W. G. and A. B. Corey. Science 82: 175, 1935.
(216) Wyckoff, R. W. G. and A. B. Corey. Proc. Soc. Exper. Biol. and Med. 34: 285, 1936.

PHYSIOLOGICAL REVIEWS

VOL. 19 JULY, 1939 No. 3

THE EFFECTS OF EXTIRPATIONS ON HIGHER BRAIN PROCESSES

C. F. JACOBSEN

Washington University School of Medicine, St. Louis, Mo.

Observation of behavioral adjustments after injury to the nervous system has been, for more than a century, one of the principal sources of knowledge concerning the functions of the brain. Refinements of the extirpation technique, introduced in recent years through the development of neurosurgery and by elaboration of the earlier methods of studying the behavior of animals, have made possible new experimental studies of the correlation of behavior and neural structure. With this development has come recognition of the need for knowledge of the behavior mechanisms of the normal subject in order to understand the nature and significance of defects associated with cortical injuries. Comparison of defects associated with occipital injury in rat, monkey, and man may be seriously misleading because of the different rôles played by vision in their normal action systems. The almost constant visual alertness in primates is in contrast to apparent lack of visual dominance in the rat. Studies of sensory control of the maze habit further indicate the lack of sensory dominance among the several sensory avenues. Lashley (1938, p. 180) has emphasized a further difference in the apparent dominance of remote over local visual cues and points out that "the conditions of visual attention must be *quite different in the rat from those in man*" (*without detracting* thereby from the many similarities of visual organization in rat and in man). Closely allied to the question of sensory dominance is the "encephalization of function"—the progressive shifting of function, in the phyletic series, from older to more recently developed nervous structures and by the increasing importance of the higher centers in the regulation of behavior (Dusser de Barenne, 1934; Fulton and Keller, 1932; Marquis, 1934). While the concepts of sensory dominance and encephalization of function are too general for application to a specific

instance, except as it may be experimentally established, attention to these principles may contribute to better evaluation of a particular experiment.

One of the outstanding changes in our views of the functions of the cerebral cortex during the past decade has been recognition of the close interdependence of the cortical and subcortical structures in the mediation of behavior. Not only have behavioral modifications, formerly thought to be characteristic of cortical function, been shown to occur at subcortical levels, but many presumably lower center instinctual functions have been found to be significantly influenced by cortical lesions. Because of this change in emphasis, studies relating to the subcortical mechanisms and learning at "simple" sensory-motor levels, as well as to behavior at the traditionally "higher" levels, have been considered in some detail.

Response to brightness differences. Knowledge of the neural correlates of optically induced behavior has increased greatly since Munk's localization of a visual center in the occipital lobes, and the formulation of experimental problems has changed from a rather futile attempt to determine whether a given bit of behavior involves "seeing" to the investigation of the neural and stimulus control of measurable visual capacities. It is clear from experiments with varying behavioral methods and on a variety of animal subjects that in the absence of the occipital areas certain responses to optical stimulation, other than the ocular reflexes, remain intact or, if disturbed by the lesion, can be reacquired.

Three series of experiments with essentially comparable methods have demonstrated that discrimination of differences in intensity of two stimulus patches is possible after complete bilateral destruction of the striate areas (in the rat, Lashley, 1935b; in the dog, Marquis, 1934; in the cat, Smith, 1937b). Using a conditioned lid response to an increase in intensity, Marquis and Hilgard (1936, 1937) have demonstrated the capacity of the dog and monkey to respond to changes in light intensity after removal of these areas. Poltyrew and Zeliony (1930) and Culler and Mettler (1934) have reported establishing simple conditioned responses to sound and light in decorticated dogs. Kluver (1936) has reported evidence of response to changes in intensity after removal of the occipital lobes in monkeys and was further able to show that response occurred to a sudden decrease in light as well as to an increase—the condition to which the animal had originally been trained. It has not thus far proven feasible to apply equally rigorous testing

techniques to the problem of response to brightness in man after bilateral occipital lobectomy, but Marquis (1934) after a review of clinical materials concluded that "destruction of the visual area of the cerebrum in man results in a complete loss of all visual sensations and of all optic reflexes with the exception of pupillary constriction" (p. 574). With possible exception of man and the anthropoid apes, which have not been examined, it is clear that animals lacking the visual cortex can form habits based on certain aspects of optical stimulation.

Two problems arise from these observations: 1, the characteristics of visually controlled behavior in animals lacking the striate areas, and 2, the other cortical or subcortical structures concerned in the mediation of these habits. Lashley's observations on the rat indicate that in addition to response to intensity differences, a sort of primitive detail vision—the ability to distinguish differences between two masses of light—is retained. Smith and Marquis have reported similar results for the cat and dog. In the monkey (Kluver, 1936) attempts to demonstrate response to position or direction of light masses have given no indication of retention of this ability after complete occipital lobectomy; either increase or decrease in intensity of the test objects or in the general illumination of the room were the only aspects of optical stimulation which evoked differential responses.

Threshold determinations for the discrimination of brightness differences between two lights of unequal intensity have been made in the rat (Lashley, 1930), in the dog (Marquis, 1934) and in the cat (Smith, 1937). Thresholds in the dog and cat, obtained under conditions of minimal illumination of the surrounds of the stimulus masses have shown a slight but not very significant increase after complete extirpation of the striate areas. Tests on the rat under diffuse daylight conditions indicated rather large increases in threshold values for the operated animals. A possible explanation for this difference is found in Smith's experiments on cats, which were tested under three conditions of the surrounds: minimal illumination, low general illumination and high general illumination. There was no marked variation in the ability of the normal animal to discriminate differences in intensity of the stimulus patches under conditions of low and high general illumination. On the other hand, extirpation of the striate areas permanently impaired ability to make discriminations under conditions of high general illumination. While the evidence clearly shows that responses to differential brightness cues can be established in the absence of the occipital areas and may under favorable circumstances appear to be not

inferior to those found in the normal animal, the loss of the geniculo-cortical system places certain limitations on the functional capacity of the remaining visual mechanisms.

While these limitations in the discrimination of brightness by the animal lacking the striate cortex have been emphasized, there are indications that in some respects the organization of visual activity related to brightness is not greatly different in the normal and operated animals. Hebb (1937 a, b; 1938) has presented evidence showing that the figure-ground relationship, the perception of identity in certain geometrical figures and the transfer of response in the discrimination of brightness and size are innately determined. In the normal animal discrimination of brightness appears to be relative rather than absolute. When subjects trained to select the more intense of two stimuli are confronted with hitherto unexperienced intensities, they react on a relative rather than an absolute basis even though in doing so it may require rejection of the previously positive stimulus. This characteristic of brightness discrimination in not significantly altered in animals in which the geniculo-striate system has been damaged or completely destroyed. Capacity for relational response is not restricted to cortical tissue.

In still another respect the brightness discrimination habit in the normal and operated subjects may be indistinguishable. Rate of acquisition of the habit in subjects lacking the striate area is not significantly different from normal animals as measured by trials and errors made in learning. (Marquis' observations on the dog suggest postoperative retardation but probably not of significant magnitude. See also Ghiselli, 1938.) Similarly acquisition of the conditioned lid response to changes in intensity of illumination, except for a slight increase in the latency of response, was not measurably different in normal and operated dogs and monkeys.

This apparent normality of acquisition in the absence of the striate areas, when considered in relation to postoperative amnesia after destruction of this region in animals that learned the brightness habit before operation, presents a perplexing problem which has not as yet found a satisfactory explanation. Tests of postoperative retention of the reaction to light in the Yerkes box (Lashley, 1935b) have been consistent in showing complete loss of the habit after destruction of the striate cortex, a finding that has also been confirmed in the cat and dog (Smith and Marquis). The habit is somehow dependent on the striate cortex when learned by the intact subject yet in the absence of

this region it can be acquired within normal limits of training (cf. Ghiselli, 1938). While retention of the habit is in some manner dependent on the striate cortex Lashley's evidence shows that it is not dependent on any particular part of this area. As long as any part of geniculo-striate system, even a few cells connected with the nasal retina, remains intact, the brightness habit may be retained without loss.

Results with the conditioned lid response to changes in intensity have given somewhat different results than those obtained with the Yerkes discrimination box. Marquis and Hilgard (1936) found perfect retention after destruction of the occipital lobes in the dog. They conclude: "Two lines of evidence support the view that the brain mechanism mediating the conditioned response is the same in normal dogs as after removal of the visual cortex: (1) the similarity of all characteristics of the conditioned response before and after operation, and (2) the retention without retraining of a conditioned response acquired prior to operation. The results indicate, not that vicarious functioning has taken place, but that the normal neural mechanism of conditioning has been practically undisturbed by removal of the occipital lobes. Evidence is presented that the essential locus of this neural mechanism is subcortical" (p. 177). Findings on the monkey, however, were equivocal in that two animals showed partial amnesia and one subject good retention of the habit (1937).

This difference in findings may be, in part at least, a function of the different demands on the organism made by the discrimination box and conditioning techniques. The discrimination box required response to the direction of stimulation (possibly a primitive form of pattern vision) *as well as response to brightness whereas the conditioning method* required response only to changes in intensity without regard to direction (cf. Kluver, 1936 and above). Also different motor mechanisms are involved since the discrimination box utilizes the greater part of the skeletal musculature while the conditioned eye-lid technique requires only the restricted musculature of the corneal reflex. The significance of these factors and the identification of the subcortical centers concerned in these responses remain to be determined.

Response to visual detail. A similar situation appears to prevail with respect to mechanisms of detail vision. While it is clear that for certain aspects of detail vision the intactness of the striate cortex is important, Smith (1938) has questioned the current emphasis on the striate cortex as the sole neural mechanism concerned. In support of

this position he considers his own observations (1937a, 1938) and those of Ter Braak (1936), which show that removal of the striate cortex in several infra-human mammals does not significantly alter the form of optic nystagmus evoked by complex patterns (alternating black and white bands). Similarly the optic nystagmus produced by apparent movement remains after loss of the occipital lobes. Tests for visual acuity, using the optokinetic responses as indicator, show no superiority of the geniculo-striate system over the subcortical mechanisms involved in these reactions. One important difference has been noted, however, in that the animal lacking the striate cortex fails to respond to a single line or to widely separated stripes. It is suggested that this loss is a specific defect in visual capacity associated with impairment of the central mechanisms for foveal vision. Smith and Bojar (1938, p. 211) conclude: "The view that the cortical centers of vision are the only neural centers sufficiently organized to mediate a highly refined visual acuity is refuted by the results and interpretations presented above."

While these forced reactions to moving visual patterns may partake of some of the characteristics commonly associated with pattern vision, it is clear that the geniculo-striate system is essential to the more varied adjustments involving response to visual detail and indeed to at least one aspect of optic nystagmic response. With the development of adequate techniques for examining detail vision in the rat, Lashley (1931) and Lashley and Frank (1934) have shown visual memory, as tested by differential response to inverted and upright triangles, is not conditional on any extra-striate area or on any part of the striate area other than the projection field of the temporal retina. While damage to this cortical field may result in disturbance of the habit, perfect retention can in some instances be obtained if a part of the superior or inferior temporal projection in one hemisphere remains intact. However, within the projection field of the temporal retina there is no evidence of localization of the habit. Evidence has also been presented that the visual cortex is essential to perception of distance as measured by force exerted in jumping various distances (Lashley, 1937). Spence and Fulton (1936) and Poljak and Hayashi (1936) have shown that in primates visual acuity suffers a slight impairment with complete extirpation of one striate area and failure to discriminate after destruction of the macular area and extensive damage to the peripheral projection areas of the second hemisphere. There was indication, however, after the second operation that the animal could respond to the position of the two test fields, to light and to movement of objects in parts of the visual

field. Kluver's observations (1936) indicating that after complete bilateral occipital lobectomy monkeys fail to utilize visual cues in orienting themselves in space or in manipulating their environment have already been discussed. His studies involving subtotal lesions of *the occipital lobes (1937) demonstrate a striking retention of visual* function. The test situations include measurement of responses to differences of brightness, color, form and size, responses to sound, weight discrimination and reaction times. When cases with unilateral lesions of the occipital lobes were examined postoperatively, it was found that *differential responses to the above tests were either not lost at all or* disturbed only slightly. In the few instances of loss, the preoperative level of performance was quickly reached. Postoperative thresholds were similar to those obtained before operation. No serious limitation of ability to "generalize," i.e., to detect similarities in heterogeneous situations, was observed.

Bilateral destruction of the macular regions resulted in apparent blindness for the first few days. By the fourth week the utilization of visual cues in reaching for and grasping food or other objects and in responding to people in the room was not markedly different, except for field defects, from the behavior of normal monkeys. During the first days of experimental testing the differential responses to visual stimuli were disturbed under some conditions. At the end of four weeks the differential responses established preoperatively were again intact. Threshold determinations involving color and form were not altered. On the other hand, performances were not entirely errorless and variations in the test situations disturbed the subject to a greater degree than before operation. The surprising feature of the experiments is the degree to which visually directed behavior resembled that of normal monkeys.

Responses to auditory stimuli. The experimental studies of learning based on other sense modalities are less complete than those in the field of vision. The earlier observations of Munk and of Rothmann, that complete bilateral destruction of the auditory areas is essential to induce serious impairment, have been confirmed by several investigators. The experiments of Wiley (1932, 1937) and Pennington (1937, 1938) reveal a marked similarity between the acquisition of simple auditory discrimination habits and the learning of the brightness habit in rats. Wiley has shown that initial postoperative acquisition of a differential response to noise is not significantly inferior to the rate of learning by a normal subject. On the other hand, postoperative retention is seri-

ously impaired by such lesions. Pennington similarly found that a learned differential response involving localization of the sound source was lost after extensive injury to the lateral surface of the brain. Initial postoperative acquisition of the response was not affected by lesions of the auditory areas. Extensive lesions in other areas of the cortex did not influence learning or retention. The critical cortical area, as determined by these behavioral techniques, was found to coincide with the cortical projection field of the medial geniculate nucleus. There was no evidence of subordinate specialization within this region. Odani (1935), failing to find localization of a similar response to noise, concluded that retention of the habit was due to the primary activity of the subcortical centers. The more carefully controlled experiments of Wiley and Pennington seem conclusive, however, in indicating the participation of the cortex in the intact organism.

The experiments of Girden, Mettler, Finch and Culler (1936) on retention and acquisition of conditioned responses to acoustic, tactile and thermal stimulation after complete decortication are of particular significance. In the normal dog or in one with extensive cortical injury, the first form of conditioning involves a diffuse response and it is only later that the localized adaptive response (withdrawal of paw) develops. In the fully decorticate dog this diffuse conditioning develops as rapidly as in the normal but the localized adaptive response cannot be obtained in the decorticate specimen. Development of this highly differentiated response appears to depend on the presence of cortical structures. (Conditioned responses were established to acoustic, thermal and tactile stimuli.) A differential response to acoustic stimuli, with maintenance of a vigorous response to a bell during gradual decline in response to a 1000 $\sim$ tone, and extinction and reestablishment of the conditioned response to a loud tone, were demonstrated.

Sensory discrimination. The progressive corticalization of sensory function in primates, as measured by tests for discrimination of weight and surface texture, has been investigated by Ruch and Fulton (1935) and Ruch, Fulton and German (1938). In the monkey, no disturbance of weight discrimination ability was observed after lesions of the postcentral gyrus and the posterior parietal lobules. Lesions involving both of these regions resulted in some permanent deficit although not complete abolition of the ability. Similar tests with the chimpanzee indicate some initial postoperative impairment which disappeared with retraining. In clinical cases, studied with similar methods for thresh-

old determinations, the initial disturbance of function was greatly ameliorated through specific training although a residual deficit remained. The clinical and experimental findings are in agreement in indicating that the capacities concerned are not focally represented in the parietal lobe and that after subtotal lesions a surprising degree of recovery can be brought about through specific reëducation. The structures involved in this recovery, whether the motor areas, remaining portions of the parietal lobe, or the thalamus, are not at present known.

Olfactory discrimination. The mechanisms involved in learning and retention of olfactory discrimination by the rat differ considerably from those of the other sense modalities in that the cerebral cortex is not directly concerned. Swann's (1934, 1935) striking results indicate that destruction of various parts of the archipallium, including the hippocampal complex, pyriform lobes, septum, and amygdaloid nucleus, and interruption of the main fibre tracts to and from the archipallium—although not complete in any one animal—resulted in no interruption of olfactory discrimination. Dependence on particular structures could be demonstrated for only the anterior limb of the anterior commissure and the olfactory bulbs. Brown and Ghiselli (1938) have shown that unless all incoming fibres were lost, olfactory discrimination could be established. Ablation of the olfactory bulbs resulted in complete loss of the habit, which demonstrates the adequacy of the technique for detecting losses of olfactory discrimination. Destruction of as much as 85 per cent of the cerebral cortex revealed no amnesia for the olfactory discrimination habit although it did produce profound sensory and motor disturbances and aimless running in the test apparatus. This period of aimless activity was rather suddenly replaced by motivated behavior, and once this change occurred, the olfactory discrimination was executed without evidence of loss. While the identification of the subcortical structures involved in this habit has not been made, the results do indicate that the cortex is not directly concerned in acquisition and retention of this olfactory discrimination. Comparable studies are not available for other animals.

The functions thus far considered have related primarily to a single sensory modality and have shown, e.g., in the rat, quite different characteristics of cerebral organization. In various instances there is dependence on a small localized area of the cortex involving spatial distribution of elements (pattern discrimination), dependence on a particular cortical area in the intact subject although the habit can be mediated through other mechanisms in the absence of the cortex (brightness

discrimination and sound localization) or, on the other hand, independence of any cortical area, even for a general facilitating background (olfactory discrimination).

Within the level of complexity for the problems involved, there was no indication of involvement of association areas apart from the primary projection areas. Problems demanding abstract visual generalizations have not been investigated although preliminary studies by Kirk (1936) with more complex patterns suggest that the discrimination habit may involve some extra-striate functions. On the other hand, a considerable body of data indicates that in the rat the primary projection areas subserve not only their immediate sensory functions but also participate in the integrating activities of the cortex in a manner that is independent of the other sensory functions. This latter type of integration shows little functional localization in the cerebral cortex, and a considerable relationship between efficiency and the mass of available cortex.

Maze learning in rats. The comprehensive studies by Lashley (1929) and Lashley and Wiley (1933) have demonstrated a consistent impairment of maze learning ability following damage to the cerebral cortex. The relationship between extent of injury and degree of deterioration was found to be curvilinear, error scores being a logarithmic function of the size of the lesion. Locus of the lesion, on the other hand, bore little relationship to retardation. Cortical destruction within the fronto-motor, somesthetic and visual areas caused approximately equal degrees of deterioration, whereas lesions in the auditory region, although they resulted in impairment, were slightly less damaging. Relative difficulty of simple and complex mazes (obtained by duplication of identical elements) was found to be the same for operated and control animals. While the authors do not regard the results as conclusive for the exact equivalence of the several areas, a considerable degree of equipotentiality of the various regions of the cortex in mediating the maze habits is indicated. In considering the above data and other studies, the authors conclude that "the uniform trend of the data . . . suggests that there must be some constant causal factor in consistency of maze performance dependent upon the mere quantity of cerebral tissue and not an artifact arising from the limitation of this or that special function" (p. 47). These results and interpretations, considered in relation to the specific sensory functions known to be mediated through the various sensory spheres, attribute to a given sensory or motor region participation in two somewhat disparate types of functions. One is the mediation of habits and functions related to the

sensory modality in question; the other is of a more general nature and is concerned with integrations above a simple sensory-motor level.

Behavioral analyses of maze performances implicate not merely one but several sensory modalities in the stimulus control of this habit and deprivation through peripheral loss of the receptor may seriously disturb performance of the maze habit. Hunter (1930, 1931; see also Lashley, 1931) while not denying other than sensory functions to a projection area, has pointed out the possibility that the deterioration in the maze performance may be due primarily to sensory deprivation—the larger lesions resulting in disturbances in several sense fields—rather than to impairment of a non-localized integrating capacity.

Tsang's experiments (1934, 1936) were designed to test this question as it relates to the most highly developed sensory projection system in the rat, i.e., the visual. If the functions of the striate areas are purely sensory, then peripheral sense deprivation and destruction of the striate areas should evoke equally severe deterioration. Such was not the case. Cortical destruction, although some visual functions could still be mediated through subcortical paths, resulted in significantly greater deterioration than enucleation of the eyes. Furthermore, the impairment after cortical injury does not seem to be due to disruption of an acquired intra- or inter-sensory organization of space habits. In Tsang's second experiments the animals were peripherally blinded before the eyes were opened and presumably deprived of any visual functioning of the striate area or of sub-cortical mechanisms. Animals thus blinded in infancy were not significantly inferior to the normal controls in acquisition and retention of the maze habit. Subsequent destruction of the striate areas caused marked retardation and the animals exhibited the behavioral characteristics of normal animals which had undergone similar cortical injury. These experiments do not exclude or deny the possibility of deterioration in maze performance being due in part to sense deprivation from cortical ablation. They do suggest, however, that the striate areas participate in non-visual integrative functions of the cerebrum although the mechanisms of such participation are not now known.

Other complex adaptations in rats. Evidence of a non-localized integrating function has been reported by other investigators on various types of problems. The delayed alternation problem (Loucks, 1931) required that the animal alternately choose the right or left pathway in a maze and imposed the further task of remembering the correct

path during a fifteen second interval between trials. Extirpation of the cortex in the fronto-motor region prior to training resulted in severe impairment in the rate at which the problem was learned and in the case of subjects operated after training, in severe amnesias which were proportional to the magnitude of the injury. Maier's experiments (1932, 1938) involved two types of behavioral organization, one involving combination of isolated experiences (reasoning) and the other association of contiguous experience (learning). The former type appears to be more susceptible to cortical injury than the latter and suggests the independence of neural mechanisms mediating the two types of behavioral organization. As in the maze experiments cited above, deterioration of "reasoning" ability was not exclusively associated with injury to a specific cortical region but followed lesions in any part of the dorsal convexity of the cerebrum. The severity of impairment was related to magnitude of cortical defect as expressed by the correlation coefficients of 0.54 and 0.75 for the anterior and posterior regions of the cerebral cortex.

Problems calling for manipulation of specific features of the environment, such as depression of a bar or tearing through a strip of paper, yield similar evidence. Retardation in rate of learning follows injury to various cortical areas. While some of this retardation can be attributed to sensory defects, the greater retardation associated with cortical injury rather than with mere sense deprivation indicates that other factors—restriction in variety of exploratory acts, in time spent in exploration and in development of manipulative patterns adapted to the specific latches—are probably operating. These latter deficiencies are characteristic of animals with extensive brain lesions without respect to the locus of the injury. Other latch box problems capable of solution without the development of specific adaptive movements are learned in normal time after extensive lesions of the cortex. Lashley (1935a) has interpreted these data as indicating "that the mechanism of association, as such, is not disturbed by cerebral lesions and that retardation from cerebral lesions is due rather to disturbance of such functions as are implied by the terms attention, insight and initiative" (p. 38).

Stereotypy of behavior after cortical injuries has been noted by various investigators (Hamilton and Ellis, 1933; Lashley, 1929; Maier, 1932). Experiments on variability and plasticity in behavior indicate similar limitations in the behavior of cortically impaired animals. The behavior of an intact animal when placed in a novel situation is charac-

terized by a series of attempted solutions directed toward various aspects of the environment. In the normal subject a given mode of behavior is persisted in for a time, then dropped and replaced by a new approach, which in turn may again be supplanted by still another attempted solution. The effect of even small cortical injury is to restrict severely the number and complexity of attempted solutions. The nature of this restriction seems in part to be a function of the sensory areas involved rather than total extent of cortical injury (Kreshevsky, 1935). In other test types the subject was confronted by a maze which allowed different but equally adequate solutions. It was found that the cortically impaired animal was not only inferior in arriving at a simple generalized solution to the problem, but it had investigated *fewer* possible solutions than the normal subject. This defect bore no relation to the locus of the lesion although severity of defect correlated with the magnitude of the injury (1937). Similar defects were observed in another problem which allowed two solutions: the one, a short path to the food which necessitated the animal's losing sight of the food for a short time; the other, a much longer path but one on which the food remained within the visual field. The normal rat was capable of adopting the former type of solution when it resulted in more efficient performance and the tendency to use this solution increased with practice. On the other hand, rats which had suffered small cortical lesions exhibited marked preference for the longer route which kept the goal in view both in the early trials and after considerable training in this problem. This decrement in adoption of "umweg" type of solution followed small cortical lesions and was not related to either locus or magnitude of cortical damage.

Studies of innate maternal behavior patterns in primiparous rats (Beach, 1937, 1938) indicate that the cortex contributes an integrating and directing component to this behavior. Many of the defects in behavior do not point to a specific sensory or motor deficit but to an inability to direct behavior toward a definite end. Nest building in the cortically operated animal begins later in the pre-partum period than does that of the normal or peripherally blinded subject and the quality of the nest, when built, is inferior. The partially decorticated mother may spend as much time as the normal animal in carrying its pups around the cage but at the end of the period the litter of the operated animal is still scattered about the cage while that of the normal mother has been collected in a nest. As in one of the maze situations, peripheral deprivation of one or another of the sensory avenues

resulted in much less disturbance of this instinctive behavior pattern than did damage to cortical tissue. No particular portion of the cortex appears more important than any other part and the severity of deficit bears some relation to extent rather than locus of the lesion.

The failure to find evidences of functional localization of more complex adaptations has been a consistent result in studies of cortical function in the rat. In these activities the cortex appears to function more as a dynamic whole than as a highly specialized structure although the theories of mass action and equipotentiality have been criticized as violating principles of structural organization of the nervous system (Mettler, 1935).

No conclusive evidence has been presented in support of the so-called association areas in the rat. This lack of evidence may reflect inadequacies of behavioral techniques and the difficulties of producing, in a brain as small as that of the rat, lesions of a discrete area without at the same time interrupting fibre tracts to areas beyond the confines of the primary cortical destruction. The anatomical studies of the primary projection fields in the rat cortex indicate adjacent regions which may well be associational centers that have thus far escaped detection.

Higher levels of organization in primates. The experimental and clinical studies of the primates leave little doubt concerning the existence of specialized fields for the elaboration of more complex aspects of behavior. The temporo-parietal and frontal regions appear to be of special significance, and injury to these regions results in particular kinds of behavioral impairment. Within the more strictly motor portions of the frontal lobes, lesions of the premotor areas have been observed to disturb the ordering of a series of acts although it leaves the execution of isolated movements relatively intact (Jacobsen, 1934). Richter and Hines (1938) indicate that injury to a different region, Brodmann's area 9, is accompanied by increased spontaneous activity.

The chimpanzee or monkey which has suffered extensive injury to the portions of the frontal lobe anterior to those just discussed shows other changes in behavior (Jacobsen, Wolfe and Jackson, 1935; Jacobsen and Nissen, 1937). The normal chimpanzee has considerable facility in using sticks or other objects to manipulate its environment, e.g., to reach a piece of food beyond its unaided reach. It can solve such problems when it must utilize several sticks, some of which may not be immediately available in the visual field. After ablation of the pre-frontal areas, the chimpanzee continues to use sticks as tools but it may have

difficulty in solving the problem if the necessary sticks and the food are not simultaneously present in the visual field. It exhibits also a characteristic "memory" defect. Given an opportunity to observe a piece of food being concealed under one of two similar cups, it fails to recall after a few seconds under which cup the lure has been hidden and it is as likely to select the cup containing no food as the correct cup (delayed response test). Capacity to perform the delayed response test has been shown in monkeys to depend on the intactness of a small portion of the frontal lobes. Extensive injuries to other parts of the cortex, the motor and premotor areas, parietal and temporal lobes, postcentral gyrus and incomplete lesions of the occipital lobes, caused no impairment of this capacity although such lesions might be accompanied by other disturbances (Jacobsen, Elder and Haslerud, 1936; Breslaw, Barrera and Warden, 1934; Settlage, 1937).

The deterioration of behavior is not by any means generalized but it is specific to certain situations. Many aspects of the animal's behavior are quite normal. Learning and retention of visual-kinesthetic-motor habits and of sensory discriminations are not impaired. Delayed conditioned reflexes, involving inhibition of response for ten to twenty seconds, may suffer some disturbance from frontal injury, but they are reëstablished without significant deviation from preoperative learning (Finan, 1937).

The observations of Kluver and Bucy (1938) on the effects of temporal lobectomy in the monkey present a type of disorder characteristically different from those observed after frontal, parietal or occipital lobe lesions. Motor reactions involved in walking, running, climbing and grasping were not impaired although certain other reactions were performed more slowly. Appreciation of size, shape, brightness and position were not significantly different from the normal monkey. There was but a slight visual field defect. Cutaneous sensitivity and motor reactions to various sounds were not impaired. Although there was no gross deficit in ability to discriminate visually, the ability to recognize and detect the meaning of objects by optical cues alone seemed to have been completely lost. The hungry animal picked up all objects within reach and after examination by mouth, discarded the inedible items. The same inedible object might be repeatedly picked up and discarded. Forms used in a discrimination problem apparently did not signify food but they were responded to as objects to be examined. Response to the figures did not appear to be a means to an end but an end in itself. Emotional responses were ap-

parently lost. Objects or actions which arouse fear, anger or resentment in the normal animal were accepted without sign of reaction. Although final evaluation of these results must await further anatomical study, it is apparent that they represent a type of defect not found after injury to the other cortical regions.

The fractionings of behavior observed after various injuries to the nervous system appear to have little relation to current psychological categories. Few, if indeed any, psychological functions that are derived from analysis of the activity of the whole organism are localized in a particular region of the brain. Thus, discrimination and response to relations are not peculiar to the cerebral cortex but they may also characterize the activity of subcortical centers. The defects associated with injury to the frontal lobe are not adequately encompassed by such terms as immediate memory, synthesis, temporal organization, or abstraction although these psychological functions may be modified by damage to this region of the brain. Similarly it is not of importance whether the defects described by Kluver and Bucy are classified as visual or agnostic for understanding their neurological mechanisms. At the present state of our knowledge of the inter-dependence of the so-called "elementary" and "higher" brain processes, description in experimental terms of the behavioral defects is more appropriate than elaborate psychological formulations.

SUMMARY

The cerebral cortex has long been regarded as the mechanism *par excellence* for the modification and integration of behavior, and some of the experiments here reviewed have added to our knowledge of these cortical functions. Equally significant, however, is the emphasis which must be placed on the subcortical centers in mediation of learned behavior. In the rat, learning based on some sensory modalities may be independent of the cortex, in other instances habits that are mediated through the cortex in the intact subject can be acquired with equal facility in the absence of the cortex, or, as in pattern vision, they may be dependent on the fine spatial distribution of cortical elements. Further, behavior mediated as subcortical levels is not necessarily of a primitive sort since it exhibits in some instances the same characteristics of response as cortically organized activity. Certainly the Pavlovian view which attributes modifiability exclusively to the cerebral cortex can no longer be maintained. In the higher mammals, and especially in the primates, the cortical centers have assumed a greater rôle than

in the rat, but even here it is clear that, with the possible exception of man, modification is not restricted to cortical mechanisms. The neural structures concerned in these subcortical activities and the manner in which the cortex and subcortical centers are organized remains largely unknown at present.

Development of specialized neural structures mediating the more complicated modes of behavior in the higher mammals seems to be clearly established. In the rat there is little evidence for specialized regions of elaboration other than the sensory and motor spheres, although this lack of demonstration may be due to inadequacies of method. On the other hand a number of studies have consistently indicated that the sensory and motor regions participate in a more general type of integration that is dissociated from their functions as sensory regions *per se*. Within limits all parts of the cortex appear to be equally capable of mediating this type of integration. In the primates, however, at least some of the more complicated behavioral adjustments clearly depend on the intactness of restricted areas, especially the parieto-temporal and frontal regions, and injury to these areas results in disturbances not found with damage to any of the sensory or motor projection fields.

REFERENCES

BEACH, F. A. The neural basis of innate behavior. I. Effects of cortical lesions upon the maternal behavior pattern in the rat. J. Comp. Psychol. **24**: 393, 1937.

The neural basis of innate behavior. II. Relative effects of partial decortication in adulthood and infancy upon the maternal behavior of the primiparous rat. J. Genet. Psychol. **53**: 109, 1938.

TER BRAAK, J. W. G. Untersuchungen über optokinetischen Nystagmus. Arch. néerl. de Physiol. **21**: 310, 1936.

BRESLAW, B., S. E. BARRERA AND C. J. WARDEN. The effect of removal of the postcentral convolution of the macacus rhesus monkey upon the delayed response. J. Comp. Psychol. **18**: 207, 1934.

BROWN, C. W. AND E. E. GHISELLI. Subcortical mechanisms in learning. IV. Olfactory discrimination. J. Comp. Psychol. **26**: 109, 1938.

CULLER, E. AND F. A. METTLER. Conditioned behavior in a decorticate dog. J. Comp. Psychol. **18**: 291, 1934.

DUSSER DE BARENNE, J. G. Some aspects of the problem of "corticalization" of function and of functional localization in the cerebral cortex. Assoc. Res. Nerv. Ment. Dis. Proc. **13**: 85, 1934.

FINAN, J. Unpublished Ph.D. thesis on file in Yale University Library, 1937.

FULTON, J. F. AND A. D. KELLER. The sign of Babinski. Springfield, Ill., C. C. Thomas, Pp. xi + 165, 1932.

GHISELLI, E. E. Mass action and equipotentiality in brightness discrimination. J. Comp. Psychol. **25**: 273, 1938.

Hamilton, J. A. and W. D. Ellis. Behavior constancy in rats. J. Genet. Psychol. **42:** 120, 1933.
Persistence and behavior constancy. J. Genet. Psychol. **42:** 140, 1933.

Hebb, D. O. The innate organization of visual activity: I. Perception of figures by rats reared in total darkness. J. Genet. Psychol. **51:** 101, 1937a.
The innate organization of visual activity: II. Transfer of response in the discrimination of brightness and size by rats reared in total darkness. J. Comp. Psychol. **24:** 277, 1937b.
The innate organization of visual activity: III. Discrimination of brightness after removal of the striate cortex in the rat. J. Comp. Psychol. **25:** 427, 1938.

Hunter, W. S. A consideration of Lashley's theory of equipotentiality of cerebral action. J. Gen. Psychol. **3:** 455, 1930.
Lashley on "Cerebral control versus reflexology". J. Gen. Psychol. **5:** 230, 1931.

Jacobsen, C. F. Influence of motor and premotor area lesions upon the retention of skilled movements in monkeys and chimpanzees. Assoc. Res. Nerv. Ment. Dis. Proc. **13:** 225, 1934.

Jacobsen, C. F., J. B. Wolfe and T. A. Jackson. An experimental analysis of the functions of the frontal association areas in primates. J. Nerv. Ment. Dis. **82:** 1, 1935.

Jacobsen, C. F., J. H. Elder and G. M. Haslerud. Studies of cerebral function in primates. Comp. Psychol. Monogr. **13:** 1, 1936.

Jacobsen, C. F. and H. W. Nissen. Studies of cerebral function in primates. IV. The effects of frontal lobe lesions on the delayed alternation habit in monkeys. J. Comp. Psychol. **23:** 101, 1937.

Kirk, S. A. Extra-striate functions in the discrimination of complex visual problems. J. Comp. Psychol. **21:** 145, 1938.

Kluver, H. An analysis of the effects of the removal of the occipital lobes in monkeys. J. Psychol. **2:** 49, 1936.
Certain effects of lesions of the occipital lobes in macaques. J. Psychol. **4:** 383, 1937.

Kluver, H. and P. C. Bucy. An analysis of certain effects of bilateral temporal lobectomy in the rhesus monkey, with special reference to "psychic blindness". J. Psychol. **5:** 33, 1938.

Kreshevsky, I. Brain mechanisms and "Hypothesis". J. Comp. Psychol. **19:** 425, 1935.
Brain mechanisms and variability. I. Variability within a means-end-readiness. J. Comp. Psychol. **23:** 121, 1937; II. Variability where no learning is involved. J. Comp. Psychol. **23:** 139, 1937; III. Limitations of the effect of cortical injury upon variability. J. Comp. Psychol. **23:** 351, 1937.
Brain mechanisms and Umweg behavior. J. Comp. Psychol. **25:** 147, 1938.

Lashley, K. S. Brain mechanisms and intelligence. Chicago, Ill. Univ. of Chicago Press. Pp. xiv + 186, 1929.
Mechanism of vision: II. The influence of cerebral lesions upon the

threshold of discrimination for brightness in the rat. J. Genet. Psychol. **37**: 461, 1930.
The mechanism of vision. IV. The cerebral areas necessary for pattern vision in the rat. J. Comp. Neurol. **53**: 419, 1931a.
Cerebral control versus reflexology. A reply to Professor Hunter. J. Gen. Psychol. **4**: 3, 1931b.
Studies of cerebral function in learning. XI. The behavior of the rat in latch box situations. Comp. Psychol. Monogr. **11**: 1, 1935a.
The mechanism of vision. XII. Nervous structures concerned in the acquisition and retention of habits based on reactions to light. Comp. Psychol. Monogr. **11**: 43, 1935b.
The mechanism of vision. XIV. Visual perception of distance after injuries to the cerebral cortex, colliculi, or optic thalamus. J. Genet. Psychol. **51**: 189, 1937a.
Functional determinants of cerebral localization. Arch. Neurol. Psychiat. **38**: 1, 1937b.
The mechanism of vision: XV. Preliminary studies of the rat's capacity for detail vision. J. Gen. Psychol. **18**: 123, 1938.

LASHLEY, K. S. AND L. E. WILEY. Studies of cerebral function in learning. IX. Mass action in relation to the number of elements in the problem to be learned. J. Comp. Neurol. **57**: 3, 1933.

LASHLEY, K. S. AND M. FRANK. The mechanism of vision. X. Postoperative disturbances of habits based on visual detail in the rat after lesions in the cerebral visual areas. J. Comp. Psychol. **17**: 355, 1934.

LOUCKS, R. B. Efficacy of the rat's motor cortex in delayed alternation. J. Comp. Neurol. **53**: 511, 1931.

MAIER, N. R. F. The effect of cerebral destruction on reasoning and learning in rats. J. Comp. Neurol. **54**: 45, 1932.
Cortical destruction in the posterior part of the brain and its effect on reasoning in rats. J. Comp. Neurol. **56**: 179, 1932.
A further analysis of reasoning in rats. III. The influence of cortical injuries on the process of "direction". Comp. Psychol. Monogr. **15**: 44, 1938.

MARQUIS, D. G. Effects of removal of the visual cortex in mammals, with observations on the retention of light discrimination in dogs. Assoc. Res. Nerv. Ment. Dis. Proc. **13**: 558, 1934.

MARQUIS, D. G. AND E. R. HILGARD. Conditioned lid responses to light in dogs after removal of the visual cortex. J. Comp. Psychol. **2**: 157, 1936.
Conditioned responses to light in monkeys after removal of the occipital lobes. Brain **60**: 1, 1937.

METTLER, F. A. Cerebral function and cortical localization. J. Gen. Psychol. **13**: 367, 1935.

METTLER, F. A., C. C. METTLER and E. CULLER. Effects of total removal of the cerebral cortex. Arch. Neurol. Psychiat. **34**: 1238, 1935.

ODANI, S. Experimental studies upon brain mechanisms in learning. III. The retention of the auditory-motor habit after cerebral injury. Jap. J. Exper. Psychol. **2**: 129, 1935.

PENNINGTON, L. A. The function of the brain in auditory localization. II. The effect of cortical operation upon original learning. J. Comp. Neurol. **66**: 415, 1937. III. Postoperative solution of an auditory spatial problem. J. Comp. Neurol. **67**: 33, 1937.

POLJAK, S. AND HAYASHI. The cerebral representation of the retina in the chimpanzee. Brain **59**: 51, 1936.

POLTYREW, S. S. AND O. P. ZELIONY. Grosshirnrinde und Associationfunction. Ztschr. f. Biol. **90**: 157, 1930.

RICHTER, C. P. AND M. HINES. Increased spontaneous activity produced in monkeys by brain lesions. Brain **61**: 1, 1938.

RUCH, T. C. AND J. F. FULTON. The effect of precentral, postcentral and posterior parietal lesions upon the performance of monkeys trained to discriminate weights. Assoc. Res. Nerv. Ment. Dis. Proc. **15**: 289, 1935.

RUCH, T. C., J. F. FULTON AND W. GERMAN. Sensory discrimination in monkey, chimpanzee and man after lesions of the parietal lobe. Arch. Neurol. Psychiat. **39**: 919, 1938.

SETTLAGE, P. H. The effect of occipital lesions on visually guided behavior in the monkey. Psychol. Bull. **34**: 765, 1937.

SMITH, K. U. The postoperative effects of removal of the striate cortex upon certain unlearned visually controlled reactions in the cat. J. Genet. Psychol. **50**: 137, 1937a.

Visual discrimination in the cat. V. The postoperative effects of removal of the striate cortex upon intensity discrimination. J. Genet. Psychol. **51**: 329, 1937b.

SMITH, K. U. AND S. BOJAR. The nature of optokinetic reactions in mammals and their significance in the experimental analysis of the neural mechanisms of visual functions. Psychol. Bull. **35**: 193, 1938.

SPENCE, K. W. AND J. F. FULTON. The effects of occipital lobectomy on vision in chimpanzee. Brain **59**: 35, 1936.

SWANN, H. G. The function of the brain in olfaction. II. The results of destruction of olfactory and other nervous structures upon the discrimination of odors. J. Comp. Neurol. **59**: 175, 1934.

The function of the brain in olfaction. III. The effects of large cortical lesions on olfactory discrimination. Am. J. Physiol. **111**: 257, 1935.

TSANG, Y. The functions of the visual areas of the cerebral cortex of the rat in the learning and retention of the maze. I. Comp. Psychol. Monogr. **10**: 1, 1934.

Ibid. II. Compt. Psychol. Monogr. **12**: 1, 1936.

WILEY, L. E. The function of the brain in audition. J. Compt. Neurol. **54**: 109, 1932.

A further investigation of auditory cerebral mechanisms. J. Comp. Neurol. **66**: 327, 1937.

THE DETOXICATION OF CARBOCYCLIC COMPOUNDS

LESLIE YOUNG

Department of Physiology and Biochemistry, University College, London

The use of the term "detoxication" in relation to the chemical changes undergone by a compound in the animal body implies that such changes bring about an abolition or diminution of toxic properties possessed by that compound. A survey of the literature on "detoxication" reveals that chemical reactions occurring in the organism are frequently classed under this heading without reference to the toxicity of the compounds involved. It has become customary to describe as "detoxication reactions" those chemical changes occurring in the body which bring about the conversion of foreign compounds of known structure to derivatives which are excreted in the urine. It is in this commonly accepted sense that the term has been used in this review. It is necessary to recognize, however, that when used in this manner the term may be a misnomer in certain instances, for it does not necessarily follow that all foreign compounds are intrinsically toxic.

The term may be misleading in another sense. In the rat, benzoic acid is largely combined with glycine. When sodium benzoate is added to the diet of young rats, growth and survival occur only if the diet provides a supply of glycine which is sufficient for both the detoxication of the benzoic acid and the building of tissue proteins (85). In a similar manner there is inhibition of growth when bromobenzene is administered to young rats on a diet which normally contains just sufficient cystine and methionine for good growth. Growth is restored when the diet is supplemented with suitable amounts of cystine or methionine (278). It is known that bromobenzene can be converted by the rat to p-bromophenylmercapturic acid (N-acetyl-S-p-bromophenyl-cysteine) and the most probable explanation of the above results is that, under the experimental conditions used, the synthesis of mercapturic acid prevents growth by creating a deficiency of sulphur-containing amino acids. Experiments such as these indicate that in some cases the conjugation of foreign compounds takes precedence over essential physiological processes. This suggests the possibility that the toxicity of certain foreign compounds may be due partly to those

very reactions which lead to the formation of so-called "detoxication products."

Different opinions have been expressed concerning the type of mechanism which is responsible for detoxication. Sherwin (235) states: "It has been necessary for the body to call to its aid a chemical defense mechanism to guard against the poisons absorbed from the gastro-intestinal tract. After many generations this chemical defense mechanism has been so perfected in its battle against putrefaction products absorbed from the intestine that it is now quite able to cope with many of the foreign organic compounds." An alternative point of view has been put forward by Quick (203) who, in considering the detoxication of benzoic acid, says: ". . . the idea is no longer tenable that these conjugations are more or less unimportant mechanisms concerned solely with the detoxication powers of the organism. If these synthetic processes are looked upon as normal and common chemical reactions made manifest because the body is applying them to a foreign substance, it is possible to perceive how a study of the conjugation of benzoic acid may help to solve various problems of metabolism which at present seems quite unrelated to hippuric acid and glycuronic acid." It should be pointed out that certain classes of compound which undergo detoxication are not known to occur among those formed during intestinal putrefaction, and also that a number of the changes to which foreign compounds are subjected in the organism are analogous to changes which occur in the course of normal metabolism. In the present state of knowledge, however, it is not possible to decide what type of mechanism is concerned in detoxication, and it may be that the types postulated by Sherwin and by Quick are both involved.

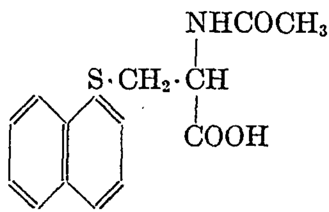
DETOXICATION REACTIONS. The chemical changes which some of the simple foreign carbocyclic compounds undergo in the animal body are reviewed below. For further information on the subject of detoxication, the excellent reviews written by Heffter (99), Sherwin (234), Ambrose and Sherwin (8), Harrow and Sherwin (98), and Quick (215) should be consulted.

Hydrocarbons. The fact that certain polycyclic hydrocarbons possess carcinogenic properties lends considerable interest to questions concerning the fate of hydrocarbons in the animal body. It has long been known that this class of compound undergoes oxidation in the body, for as far back as 1867 Schultzen and Naunyn (232) demonstrated that benzene can be converted to phenol by man and the dog. The oxidation does not stop at this stage, for catechol and quinol are also

formed (179). Other hydrocarbons undergo similar changes, and in consequence of this, questions concerning the detoxication of hydroxy-compounds are involved to a considerable extent in the study of the fate of cyclic hydrocarbons in the organism. That some of the phenol formed from benzene is excreted in a combined form was recognized by Munk (171), and it was later shown that this phenol is conjugated partly with sulphuric acid (16) and partly with glucuronic acid (143, 144). The tendency of phenolic compounds to combine with sulphuric acid disturbs the normal ratio of inorganic to organic (or ethereal) sulphates in the urine. This change has been shown to serve as an early sign of benzene poisoning in animals (285), and there is evidence that sulphate determinations on the urine are of value in the medical supervision of workers exposed to the possible risk of benzene poisoning (130). There are some grounds for believing that oxidative fission of the benzene ring can also occur in the animal body, for Jaffé (126) was able to isolate muconic acid, HOOC·CH:CH·CH:CH·COOH, from the urine of dogs and rabbits to which benzene had been administered. This was confirmed by later workers (78, 263, 59). In all cases the amounts of muconic acid obtained have been small. Jaffé considered that in the body larger amounts of benzene undergo ring fission than are indicated by the quantities of muconic acid which are excreted, for on injecting muconic acid he was able to recover only about 1 per cent of it from the urine. Similar experiments by other workers (164, 184, 59), however, have shown that a large proportion of an injected dose of muconic acid is excreted unchanged. Although benzene might be expected to give cis-cis muconic acid on oxidation in the organism (25), the form isolated is the trans-trans isomer (59). The question arises as to whether muconic acid is formed directly from benzene, or as a result of a metabolic disturbance produced by the hydrocarbon. Drummond and Finar (59) showed that both the trans-trans form of muconic acid and the cis-cis isomer are largely excreted unchanged after injection into rabbits. They observed, however, that the cis-cis isomer is partially converted to the trans-trans form when it is added to the urine of rabbits receiving benzene, although no such change occurs when it is added to normal urine. When the cis-cis acid is administered to a rabbit receiving benzene, only the trans-trans isomer can be isolated from the urine. Although these findings await explanation, they suggest that if cis-cis muconic acid is formed in the metabolism of benzene it may undergo conversion to the trans-trans form.

The metabolism of naphthalene is of especial interest in view of the

fact that when it is administered to rabbits in repeated doses a degeneration of the crystalline lens of the eye takes place which resembles that observed in human senile cataract (27, 5). Although qualitative tests for the presence of naphthols in the urine excreted after administering naphthalene have yielded conflicting results in the hands of different workers (117, 61, 191, 5), some oxidation of naphthalene to naphthol does occur, for Lesnik (146) was able to isolate α-naphtholglucuronic acid from dog urine after feeding naphthalene. In 1933 Bourne and Young (29, 30), after administering naphthalene to rabbits, isolated from the urine a compound to which, on the basis of their studies of its constitution, they assigned the structure:

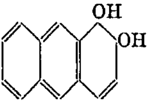


l-α-naphthylmercapturic acid

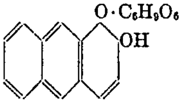
It was confirmed by synthesis (120) that the compound excreted is l-α-naphthylmercapturic acid. Its isolation removed the doubts which until then existed concerning whether or not hydrocarbons share with certain halogenated benzene compounds the property of being converted to mercapturic acids in the animal body. It has subsequently been shown by Stekol (247, 251) that other animals can synthesize l-α-naphthylmercapturic acid from naphthalene. It has recently been found that anthracene also gives rise to a mercapturic acid in the organisms of the rabbit and the rat (33) and the increased neutral sulphur excretion which follows the administration of benzene (37, 249) and phenanthrene (249) to the dog suggests that these hydrocarbons also yield derivatives of the same type. Baumann and Herter (16) obtained naphthalene by the steam distillation of the acidified urine of dogs to which naphthalene had been fed, and Bourne and Young (28, 30) observed that naphthalene is liberated from a soluble precursor by acidification of the urine of rabbits receiving naphthalene. Similar observations have been made with other animals (254). Although, in the rabbit, the compound which yields naphthalene on acidification is one of the main detoxication products of this hydrocarbon (28), all attempts to isolate it have so far failed.

We owe our knowledge of the metabolism of anthracene to the recent

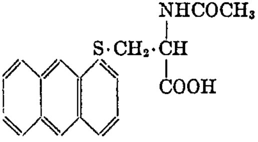
work of Boyland and Levi (31, 32, 33). They showed that the rat and the rabbit on a diet containing anthracene excrete 1,2-dihydroxy-1,2-dihydroanthracene. Whereas the urine of the rabbit contains the d-form of this compound, the rat excretes the l-form (31). The rabbit also excretes d-1,2-dihydroxy-1,2-dihydroanthracene-1-glucuronic acid, and it is probable that the rat excretes the analogous laevorotatory compound (32). Some anthracene is converted to 1-anthrylmercapturic acid by both the rat and the rabbit (33). In addition to these compounds, the urine of the rat and the rabbit, on a diet which includes anthracene, contains a compound not yet isolated, which yields anthracene on acidification (33).



1,2-Dihydroxy-
1,2-dihydro-
anthracene



1,2-Dihydroxy-
1,2-dihydro-
anthracene-
1-glucuronic acid



1-anthrylmercapturic acid

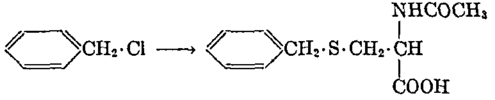
Evidence has been put forward by Bergel and Pschorr (22) that when phenanthrene is administered to the rabbit, phenanthrolglucuronic acid is excreted.

When either toluene (232, 138) or ethylbenzene (232, 262) is ingested it is oxidized to some extent to benzoic acid. There is no evidence of mercapturic acid formation after feeding toluene (37). Part of the dose of ethylbenzene is converted to phenylmethylcarbinol, $C_6H_5 \cdot CH(OH) \cdot CH_3$, and part to mandelic acid, $C_6H_5 \cdot CH(OH) \cdot COOH$. The former derivative is excreted in conjugation with glucuronic acid (262). Although n-propylbenzene, $C_6H_5 \cdot CH_2 \cdot CH_2 \cdot CH_3$, gives rise to benzoic acid in the body, this does not appear to occur in the case of isopropylbenzene, $C_6H_5 \cdot CH(CH_3)_2$, in which the side chain is branched. There is evidence that this compound is converted to a phenolic derivative (179). When two methyl groups are substituted in the benzene nucleus, as in m-xylene, $C_6H_4(CH_3)_2$, one is oxidized to a carboxylic group (80). In a similar manner, one methyl group of symmetrical trimethylbenzene (mesitylene), $C_6H_3(CH_3)_3$, is oxidized (176), as also is the methyl group in position 4 of 1:2:4 trimethylbenzene (pseudo-

cumene) (121). The changes which alkyl substituted benzene derivatives undergo in the body do not appear to be confined to the oxidation of substituent groups. For example, it has been reported (52) that mesitylene is not only oxidized to mesitylenic acid, but is also excreted partly as mesitol, $(CH_3)_3C_6H_2 \cdot OH$, and partly as p-hydroxymesitylenic acid, $(CH_3)_2C_6H_2(OH)(COOH)$. When triphenylmethane, $(C_6H_5)_3CH$, is fed to the rabbit and the dog, part of it can be recovered unchanged from the urine (163).

Halogenated hydrocarbons. The detoxication of certain halogenated benzene compounds has attracted much attention because of the effect of these substances on sulphur metabolism. It was first established by Baumann and Preusse (17, 19) that when bromobenzene is administered to the dog, part of it is transformed to p-bromophenylmercapturic acid, $Br \cdot C_6H_4 \cdot S \cdot CH_2 \cdot CH(NH \cdot CO \cdot CH_3)COOH$. Recently it has been shown that in the rat p-bromophenyl-cysteine (257), as well as bromobenzene (278), gives rise to p-bromophenylmercapturic acid. It is also known that chlorobenzene (125) and iodobenzene (21) are converted in the animal body to p-chloro- and p-iodo-phenylmercapturic acid respectively. The corresponding fluorbenzene derivative has not been isolated, but from the work of Coombs (47) it seems probable that it is formed when fluorbenzene is fed to the dog. According to Baumann (15) p-bromophenylmercapturic acid is present in the urine as an unstable complex with glucuronic acid which is easily split by dilute acids and alkalies. This derivative has not been isolated. Some bromobenzene is oxidized in the body to p-bromophenol and it is probable that o-bromophenol is also formed (19). Chlorobenzene gives rise to similar oxidation products (101). The phenolic derivatives so formed are excreted partly as ethereal sulphates and partly in conjugation with glucuronic acid. The fact that the mono-halogen substituted benzenes yield the corresponding p-substituted phenols in the organism has led certain workers (216, 240) to assume that this is a preliminary step in the synthesis of mercapturic acids. Experiments in which p-chlorophenol (48) and p-bromophenol (279) have been fed to animals have failed to reveal evidence of mercapturic acid formation. Results of this type, however, can only be regarded as indicative, for it does not follow that the changes which are undergone by a compound produced in the tissues are invariably the same as those undergone by that compound when it is administered to the animal. The negative results described above do not justify, therefore, a denial (48) of the possibility that phenol formation is a stage in the production of mercapturic acids in the or-

ganism. Some doubt exists concerning the detoxication of compounds of the dichlorobenzene type. No mercapturic acids have been isolated after administering these compounds to animals, although Baumann (15) obtained evidence of mercapturic acid formation after feeding o-dichlorobenzene. In the case of m- and p-dichlorobenzene he observed an increased excretion of ethereal sulphates, but could detect no mercapturic acid in the urine. The possibility that o- and m-dichlorobenzene may be converted to mercapturic acids in the dog is suggested by the observation of Callow and Hele (37) that these compounds have an effect on sulphur metabolism which is similar to that found in the case of monochlorobenzene. The nuclear substituted halogen derivatives of toluene tend to be oxidized in the body to the corresponding carboxylic acids (109) and no evidence of mercapturic acid formation has been found (15, 37). On the other hand, Stekol (258) recently isolated N-acetyl-S-benzyl-cysteine from the urine of dogs to which he had fed benzyl chloride:



This is the only mercapturic acid so far obtained in which the sulphur of the acetylcysteine residue is not attached directly to a carbocyclic ring.

Phenols. When phenol, $C_6H_5 \cdot OH$, is administered to an animal, part of it is conjugated, part of it is oxidized, and part of it is excreted unchanged. Baumann (12) was the first to recognize that phenol is converted to a sulphuric acid derivative in the animal body. He isolated this compound in the form of its potassium salt, $C_6H_5 \cdot O \cdot SO_3K$, and confirmed its structure by synthesis (13). Later it was shown that part of the phenol is excreted in combination with glucuronic acid (144). This derivative, $C_6H_5 \cdot O \cdot C_6H_9O_6$, is a β-glycoside (223, 182). It is known that phenolic compounds are formed as a result of the action of intestinal bacteria on protein decomposition products (35, 277, 14), and it is generally accepted that these compounds give rise to part of the ethereal sulphates present in normal urine (148). It was long considered that the production of ethereal sulphate takes precedence over glucuronide formation in the detoxication of phenols. This is not supported by the observations of certain workers (157, 222) who have

reported the presence of glucuronide in urines containing considerable amounts of inorganic sulphate. It is unfortunate that few quantitative data are available concerning the extent to which glucuronide formation takes place in the detoxication of phenols, for there is evidence (268) that man excretes more phenol in conjugation with glucuronic acid than with sulphuric acid. In the dog, phenol is oxidized to some extent to quinol and catechol (18) and these derivatives are excreted as ethereal sulphates (18) and as glucuronides (144). The methyl substituted phenols tend to be oxidized to the corresponding carboxylic acids, e.g., p-cresol, $HO \cdot C_6H_4 \cdot CH_3$, is partly converted to p-hydroxybenzoic acid, $HO \cdot C_6H_4 \cdot COOH$ (196). The extent to which phenolic compounds undergo conjugation in the organism varies considerably with the nature and position of substituent groups in the carbocyclic nucleus. Williams (283) has studied the excretion of ethereal sulphates by the rabbit after the administration of phenol and a number of its mono-substituted derivatives. Compared with phenol itself, decreased conjugation is observed when an acidic group is present in the o-position, and increased when a basic group is present in this position. In the case of a neutral group in the o-position, the extent to which conjugation occurs is similar to that observed with phenol. The m-derivatives yield similar, though less marked, results to those obtained with the corresponding o- substituted derivatives of phenol. Although in most cases substitution in the p-position has little effect on the amount of ethereal sulphate formed, sulphate conjugation is markedly decreased by a p-COOH group and increased by a p-$CONH_2$ group. In these experiments no derivative tested was found to undergo sulphate conjugation to the extent of more than 40 per cent.

Following the development and application of their colorimetric method for determining urinary phenols, Folin and Denis (71) reported that a considerable proportion of the phenols present in urine is in the unconjugated form. In the urine of a patient undergoing benzene treatment for leukemia they found that the free phenols predominated. They stated that in normal urine 30–90 per cent. of the total phenols are in the free form, and Dubin (60), who used their method for determining phenols, reported similar results. In considering these findings it is necessary to recognize that the Folin-Denis "phenol" reagent gives a blue colour not only with phenols, but also with aromatic hydroxy-acids and other substances present in urine (267). By using a method for determining phenols which involved ether extraction of the

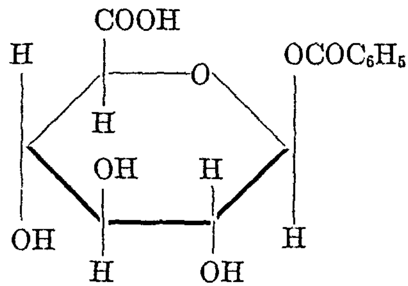
urine, Tisdall (267) obtained results for the excretion of total phenols in normal urine which are at least 50 per cent lower than those reported by Folin and Denis. He also observed that only a very small proportion of the volatile phenols (phenol and p-cresol) is present in the free state.

The work of Marrian and his co-workers (44, 42, 43, 188) and of Venning and Browne (273) has revealed that the process of excretion in conjugation with glucuronic acid is not restricted to the detoxication of foreign organic compounds. From human pregnancy urine Cohen and Marrian (43) have isolated oestriol glucuronide, and Venning and Browne (273) have obtained pregnandiol glucuronide. In the former compound the glucuronic acid is apparently not linked to the phenolic hydroxyl of the oestriol (43). There is present in mare's urine a combined oestrogen which appears to be a sulphuric acid derivative (227).

Carboxylic acids. Since considerations of space make it impossible to review here the vast amount of work which has been published on the detoxication of carboxylic acids, the present review has been based on the more recent investigations on this subject. For information on the earlier work in this field, the monograph by Dakin (56) and the reviews by Sherwin (234) and by Heffter (99) should be consulted.

Although benzoic acid has been found to undergo conjugation with glycine in every mammal so far tested, it is now known that in man (55, 183), the sheep (153), pig (51), dog (34, 200) and rabbit (84), it gives rise to a glucuronic acid derivative as well as hippuric acid. The structure of this derivative has been the subject of some controversy. Some workers (153, 197) were led to the conclusion that the compound is 1-benzoylglucuronic acid, whereas others (200, 213, 156) considered that the benzoic acid is attached to the glucuronic acid by an ester linkage to a carbon atom other than the aldehydic carbon atom. The question now appears to have been settled by the recent work of Goebel (82). As a result of the preparation of 1-bromo-2,3,4-triacetylglucuronic acid methyl ester (83), a pyranose derivative with the β-configuration (115), it is now possible to prepare derivatives of glucuronic acid substituted on the aldehydic carbon atom. By treating the above bromo-compound with silver benzoate, Goebel (82) prepared 1-benzoyl-2,3,4-triacetylglucuronic acid methyl ester and showed that this derivative is identical with the product obtained by acetylation of the methyl ester of the benzoic acid-glucuronic acid compound isolated from dog urine after feeding sodium benzoate. The benzoic acid

derivative which is excreted is therefore 1-benzoylglucuronic acid, and it has been assigned the structure:



The relative extent to which glycine and glucuronic acid are involved in the detoxication of benzoic acid varies considerably from species to species. In the dog, as much as 75 per cent of a dose of sodium benzoate may be excreted as benzoylglucuronic acid (203), whereas in man only 10–12 per cent of the dose is excreted in this form (205). The remainder of the dose in both cases is excreted almost entirely as hippuric acid. With reference to these species differences, Quick (206) has studied the formation of hippuric acid and benzoylglucuronic acid in the animal body from the standpoint of the law of mass action, and has concluded that the differences observed in the output of these substances depend on the factors which influence a reversible reaction. The reactions involved are:

Benzoic acid + glycine $\rightleftharpoons$ hippuric acid + water

Benzoic acid + glucuronic acid $\rightleftharpoons$ benzoylglucuronic acid + water.

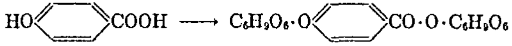
They are related in that benzoic acid is common to both of them, and Quick considers that the basic factors which determine the partition of benzoic acid between them are: (a) the rate of elimination of the end-products, hippuric acid and benzoylglucuronic acid; (b) the concentration and supply of glycine and of glucuronic acid; (c) the rates of the reactions between benzoic acid and glycine, and between benzoic acid and glucuronic acid.

It has frequently been observed that increases in the dose of a foreign compound fail to produce proportionate increases in the output of its detoxication products. For example, when increasing doses of sodium benzoate are administered to a dog on a diet containing little or no glycine, a point is reached beyond which further increases in the dose have little effect on the amount of hippuric acid excreted. On the other hand, the excretion of benzoylglucuronic acid continues to increase

with increasing doses of sodium benzoate far beyond the level at which the hippuric acid excretion becomes limited (199). It seems unlikely that conjugation with glucuronic acid is a secondary defence mechanism as was once believed, for in the dog, the formation of benzoylglucuronic acid appears to proceed at first more rapidly than the production of hippuric acid (203). Furthermore, it has been shown that in the rabbit benzoylglucuronic acid formation occurs with doses of sodium benzoate which are below those at which hippuric acid production is at a maximum (84).

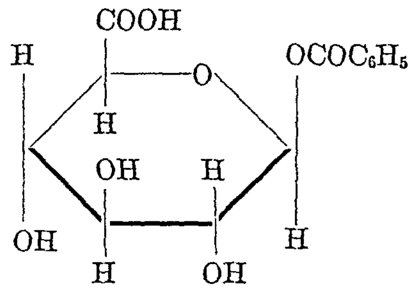
An example of the influence of the mode of administration of a foreign compound on the course of its detoxication is provided by the finding (87, 84, 271) that when sodium benzoate is injected intravenously into a rabbit, there is a greater excretion of unconjugated benzoic acid than is the case when the same dose of sodium benzoate is given orally.

The substituted benzoic acids frequently differ widely from benzoic acid in their tendency to undergo conjugation in the animal body. Thus it has been found that with the possible exception of certain o-halogen substituted acids (*c.f.* 109, 187, 208) the o-substituted benzoic acids do not combine to any marked extent with glycine in the organism. Quick (208) has shown that whereas this inhibitory action of the o-group on the conjugation of substituted benzoic acids with glycine is independent of the nature of the group, the output of glucuronic acid derivatives is decreased if there is an acidic group in the o-position, and increased if the group is a basic one. A considerable amount of attention has been paid to the detoxication of o-hydroxybenzoic acid (salicylic acid) because of the pharmacological action of this compound. Contrary to the findings of Bertagnini (24) and later workers, Hanzlik (95) concluded that it is improbable that salicylic acid is conjugated with glycine in the human or animal organism. Holmes (112) disagreed with this conclusion. More recently Quick (212) has been able to isolate salicyluric acid, $HO \cdot C_6H_4 \cdot CO \cdot NH \cdot CH_2 \cdot COOH$, in small amounts, from the urine of men receiving salicylic acid. Quick (209) has shown that the feeding of p-hydroxybenzoic acid to the dog leads to the excretion of an interesting compound in which the phenolic group and the carboxylic group of p-hydroxybenzoic acid are each conjugated with a molecule of glucuronic acid:



When p-hydroxybenzoic acid is ingested by man, it is excreted partly uncombined and partly conjugated with glycine (209).

derivative which is excreted is therefore 1-benzoylglucuronic acid, and it has been assigned the structure:



The relative extent to which glycine and glucuronic acid are involved in the detoxication of benzoic acid varies considerably from species to species. In the dog, as much as 75 per cent of a dose of sodium benzoate may be excreted as benzoylglucuronic acid (203), whereas in man only 10–12 per cent of the dose is excreted in this form (205). The remainder of the dose in both cases is excreted almost entirely as hippuric acid. With reference to these species differences, Quick (206) has studied the formation of hippuric acid and benzoylglucuronic acid in the animal body from the standpoint of the law of mass action, and has concluded that the differences observed in the output of these substances depend on the factors which influence a reversible reaction. The reactions involved are:

Benzoic acid + glycine $\rightleftharpoons$ hippuric acid + water

Benzoic acid + glucuronic acid $\rightleftharpoons$ benzoylglucuronic acid + water.

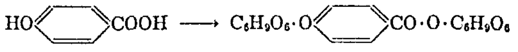
They are related in that benzoic acid is common to both of them, and Quick considers that the basic factors which determine the partition of benzoic acid between them are: (a) the rate of elimination of the end-products, hippuric acid and benzoylglucuronic acid; (b) the concentration and supply of glycine and of glucuronic acid; (c) the rates of the reactions between benzoic acid and glycine, and between benzoic acid and glucuronic acid.

It has frequently been observed that increases in the dose of a foreign compound fail to produce proportionate increases in the output of its detoxication products. For example, when increasing doses of sodium benzoate are administered to a dog on a diet containing little or no glycine, a point is reached beyond which further increases in the dose have little effect on the amount of hippuric acid excreted. On the other hand, the excretion of benzoylglucuronic acid continues to increase

with increasing doses of sodium benzoate far beyond the level at which the hippuric acid excretion becomes limited (199). It seems unlikely that conjugation with glucuronic acid is a secondary defence mechanism as was once believed, for in the dog, the formation of benzoylglucuronic acid appears to proceed at first more rapidly than the production of hippuric acid (203). Furthermore, it has been shown that in the rabbit benzoylglucuronic acid formation occurs with doses of sodium benzoate which are below those at which hippuric acid production is at a maximum (84).

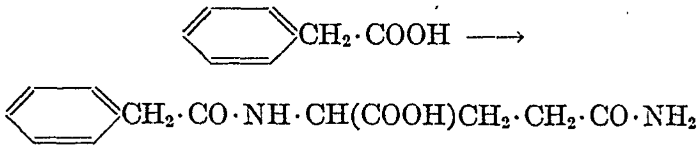
An example of the influence of the mode of administration of a foreign compound on the course of its detoxication is provided by the finding (87, 84, 271) that when sodium benzoate is injected intravenously into a rabbit, there is a greater excretion of unconjugated benzoic acid than is the case when the same dose of sodium benzoate is given orally.

The substituted benzoic acids frequently differ widely from benzoic acid in their tendency to undergo conjugation in the animal body. Thus it has been found that with the possible exception of certain o-halogen substituted acids (*c.f.* 109, 187, 208) the o-substituted benzoic acids do not combine to any marked extent with glycine in the organism. Quick (208) has shown that whereas this inhibitory action of the o-group on the conjugation of substituted benzoic acids with glycine is independent of the nature of the group, the output of glucuronic acid derivatives is decreased if there is an acidic group in the o-position, and increased if the group is a basic one. A considerable amount of attention has been paid to the detoxication of o-hydroxybenzoic acid (salicylic acid) because of the pharmacological action of this compound. Contrary to the findings of Bertagnini (24) and later workers, Hanzlik (95) concluded that it is improbable that salicylic acid is conjugated with glycine in the human or animal organism. Holmes (112) disagreed with this conclusion. More recently Quick (212) has been able to isolate salicyluric acid, $HO \cdot C_6H_4 \cdot CO \cdot NH \cdot CH_2 \cdot COOH$, in small amounts, from the urine of men receiving salicylic acid. Quick (209) has shown that the feeding of p-hydroxybenzoic acid to the dog leads to the excretion of an interesting compound in which the phenolic group and the carboxylic group of p-hydroxybenzoic acid are each conjugated with a molecule of glucuronic acid:

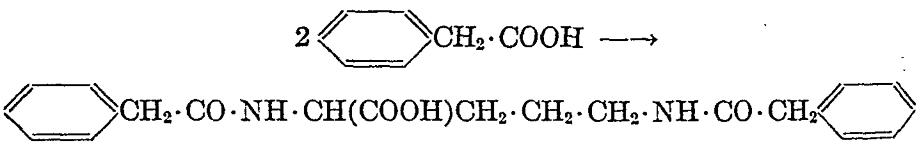


When p-hydroxybenzoic acid is ingested by man, it is excreted partly uncombined and partly conjugated with glycine (209).

In the human organism phenylacetic acid, unlike benzoic acid, does not undergo conjugation with glycine. As Thierfelder and Sherwin (264, 265) first showed, phenylacetic acid when ingested by man is largely excreted in combination with glutamine:



For a long time it seemed as though man alone is capable of bringing about this reaction. In the dog (224, 225), rabbit (225), cat (228), and monkey (233), phenylacetic acid is combined with glycine, and in the fowl (269) it is conjugated, as is benzoic acid (123), with ornithine:



Recently it has been found, however, that the chimpanzee can combine phenylacetic acid with glutamine (194). In view of these remarkable species differences, it is of interest to note that a phenylacetyl derivative of glycine, ornithine, or glutamine is largely excreted unaltered when administered to man, the dog, rabbit and fowl, irrespective of the type of conjugation which phenylacetic acid undergoes in the animal to which the derivative is given (241). Glucuronic acid is also involved in the detoxication of phenylacetic acid. In man, about 5 per cent of a moderate dose of this compound is excreted combined with glucuronic acid (7), and in the dog about one-third of the dose is eliminated in this form (203). Sherwin and his co-workers (39, 40, 169, 162, 163) have studied the fate of a wide variety of phenylacetic acid derivatives in man, the dog and the rabbit. Some of them are combined with glycine or glucuronic acid, many of them are largely excreted unchanged, but so far no compound other than phenylacetic acid has been found to undergo detoxication by union with glutamine.

Alcohols. The oxidation of primary aromatic alcohols to the corresponding carboxylic acids has been shown to occur in the body. Thus benzyl alcohol, $C_6H_5 \cdot CH_2 \cdot OH$, is converted to benzoic acid (230, 152), phenylethyl alcohol, $C_6H_5 \cdot CH_2 \cdot CH_2 \cdot OH$, to phenylacetic acid (56), and o-hydroxybenzyl alcohol, $HO \cdot C_6H_4 \cdot CH_2 \cdot OH$, to salicylic acid (175). In man and the rabbit, the oxidation of benzyl alcohol is rapid, and the

velocity of the oxidative reaction is at least as great as the rate of hippuric acid synthesis and excretion (245, 57). Some aromatic alcohols are converted to glucuronides in the body, as also are a number of aliphatic alcohols (180). This has been observed in the case of a primary alcohol, o-nitrobenzyl alcohol, formed in the body from o-nitrotoluene (124), and a secondary alcohol, phenylmethylcarbinol, formed after the administration of acetophenone (180, 262, 204). When triphenylcarbinol, $(C_6H_5)_3C \cdot OH$, is fed to rabbits, part of it can be recovered unchanged from the urine and feces (163).

Aldehydes. Most aromatic aldehydes are readily oxidized to carboxylic acids in the organism. Benzaldehyde, $C_6H_5 \cdot CHO$, given orally or by injection, is converted to benzoic acid (284, 46, 77), and in the dog may also yield small amounts of benzamide (45). The aldehydic group in other aromatic compounds such as vanillin (195) and o-, m-, and p-nitrobenzaldehydes (259), is also known to undergo oxidation in the body, and p-nitrophenylacetaldehyde, $NO_2 \cdot C_6H_4 \cdot CH_2 \cdot CHO$, in which the aldehydic group is in the side chain, is oxidized to p-nitrophenylacetic acid (237). From experiments with the aldehyde mutase of liver, it would appear that this enzyme does not act on aromatic aldehydes (189, 58).

In the presence of a suitable condensing agent, benzaldehyde and sodium acetate react *in vitro* to yield sodium cinnamate (Perkin's reaction) (192):

$$C_6H_5 \cdot CHO + CH_3 \cdot COONa = C_6H_5 \cdot CH{:}CH \cdot COONa + H_2O.$$

The fact that the heterocyclic aldehyde, furfuraldehyde, $C_4H_3O \cdot CHO$, undergoes an analogous change in the body to yield furfuracrylic acid, $C_4H_3O \cdot CH{:}CH \cdot COOH$ (127, 128), has naturally led to some speculation as to whether or not benzaldehyde is converted to cinnamic acid in the organism. No formation of cinnamic acid has been demonstrated, however, in experiments in which benzaldehyde has been given to animals by mouth or by injection (46, 77). Furfuracrylic acid can be oxidized by animals to pyromucic acid, $C_4H_3O \cdot COOH$ (72, 74), and it may be that if any cinnamic acid is formed from benzaldehyde, it is oxidized to benzoic acid. On the other hand, cinnamic acid formed from cinnamaldehyde, $C_6H_5 \cdot CH{:}CH \cdot CHO$, in the organism escapes oxidation to some extent and is excreted in combination with glycine (75).

Ketones. The mixed aliphatic-aromatic ketones tend to give rise to both secondary alcohols and carboxylic acids in the body. Acetophenone, $C_6H_5 \cdot CO \cdot CH_3$, is converted to phenylmethylcarbinol, $C_6H_5 \cdot$

$CH(OH)\cdot CH_3$, (excreted in conjugation with glucuronic acid), mandelic acid, $C_6H_5\cdot CH(OH)\cdot COOH$, and benzoic acid (177, 180, 262, 204). Benzylmethylketone, $C_6H_5\cdot CH_2\cdot CO\cdot CH_3$, is also oxidized to benzoic acid (53), and Dakin (53) has suggested that "It will probably be found that most aromatic methyl ketones primarily undergo oxidation in the body, so as to yield acids with two less carbon atoms, except in the case of acetophenone, in which the carbonyl group is directly attached to the nucleus." This is supported by the finding that phenylethylmethylketone, $C_6H_5\cdot CH_2\cdot CH_2\cdot CO\cdot CH_3$, (54, 108) gives rise to phenylacetic acid, $C_6H_5\cdot CH_2\cdot COOH$, as also does phenylbutylmethylketone, $C_6H_5\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH_2\cdot CO\cdot CH_3$, (108). This last compound would be expected first to yield phenylbutyric acid, $C_6H_5\cdot CH_2\cdot CH_2\cdot CH_2\cdot COOH$, which would then undergo β-oxidation to phenylacetic acid.

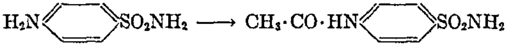
It has been observed (262) that a number of the aliphatic-aromatic ketones of the phenyl and benzyl series behave similarly to the methylketones of these series in that they give rise to benzoic acid in the body together with their corresponding secondary alcohols.

Certain aromatic ketones which are also phenolic in character are excreted in conjugation with sulphuric acid and glucuronic acid (178). p-Hydroxybenzophenone, $C_6H_5\cdot CO\cdot C_6H_4\cdot OH$, is said to be excreted unchanged (231). After the administration of o-aminoacetophenone, $NH_2\cdot C_6H_4\cdot CO\cdot CH_3$, to the rabbit, anthranilic acid, $NH_2\cdot C_6H_4\cdot COOH$, and the glucuronic acid derivative of o-aminophenylmethylcarbinol, $NH_2\cdot C_6H_4\cdot CH(O\cdot C_6H_9O_6)\cdot CH_3$, are excreted (119). These products are analogous to those obtained from acetophenone.

Amino compounds. Whereas simple aliphatic amines such as methylamine are largely destroyed in the body (132), the carbocyclic derivatives of the $R\cdot CH_2\cdot NH_2$ series are converted to carboxylic acids. Thus benzylamine, $C_6H_5\cdot CH_2\cdot NH_2$ (166, 118), and β-phenylethylamine, $C_6H_5\cdot CH_2\cdot CH_2\cdot NH_2$ (90), are oxidized to benzoic acid and phenylacetic acid respectively. Similarly, p-hydroxyphenylethylamine (66) and β-indolethylamine (67, 81) are converted to the corresponding carboxylic acids. On the other hand, Richter (217) has shown that amines of the $RR'CH\cdot NH_2$ series such as ephedrine, benzedrine and methylisomyn, are excreted unchanged by man.

A number of aromatic amino compounds have been shown to undergo acetylation in the body. When sulphanilamide (p-aminobenzenesulphonamide) is administered to man and certain animals it is excreted

partly free and partly in a conjugated form (79, 155), and Marshall *et alia* (155) have shown that in the case of man and the rabbit, the conjugated form is mainly, if not entirely, p-acetylaminobenzenesulphonamide:



This reaction does not occur in the dog (155), however, and it is therefore interesting to note that o-, m-, and p-aminobenzoic acids are excreted unchanged by the dog, whereas the m- and p-isomers are converted to the acetylamino derivatives by man and the rabbit, and only the o-form is excreted unchanged (168). Furthermore, the dog does not acetylate any of the monoaminophenylacetic acids, but excretes the o- and m-isomers unchanged and conjugates the p-form with glycine (39, 40, 169). In the rabbit all three acids are acetylated (39, 40, 169). On the other hand, acetylation of side chain amino groups has been shown to occur in the dog in the case of γ-phenyl-α-aminobutyric acid, $C_6H_5 \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot COOH$ (139), and S-benzyl-cysteine, $C_6H_5 \cdot CH_2 \cdot S \cdot CH_2 \cdot CH(NH_2) \cdot COOH$ (258).

Certain aromatic amino compounds when administered to animals are oxidized to phenolic derivatives. Thus aniline, for example, does not appear to be acetylated, but is oxidized to p-aminophenol, $HO \cdot C_6H_4 \cdot NH_2$, and excreted in conjugation with sulphuric acid (229) and possibly with glucuronic acid (143). This formation of a phenolic group also takes place in the case of β-naphthylamine, $C_{10}H_7 \cdot NH_2$, a compound which has been shown to produce bladder tumours in rabbits and dogs (106, 116). From the urine of dogs receiving β-naphthylamine, Wiley (281, 282) has isolated the sulphuric acid derivative of 2-amino-1-naphthol, the identity of which he has established by synthesis. In man acetanilide, $C_6H_5 \cdot NH \cdot CO \cdot CH_3$, is oxidized to p-acetylaminophenol, $HO \cdot C_6H_4 \cdot NH \cdot CO \cdot CH_3$ (165). In the rabbit acetanilide gives rise to p-aminophenol, which is excreted in conjugation with sulphuric acid and with glucuronic acid (129). In connection with these reactions, it is of interest to note that the liver and certain other organs of a number of animals, including the rabbit, contain an acylase which hydrolyses acetanilide *in vitro*, and also that a liver suspension can bring about the oxidation of aniline to p-aminophenol (159).

Dimethylaniline, $C_6H_5 \cdot N(CH_3)_2$, has been found to undergo demethylation in the dog and the rabbit. In the dog it is completely demeth-

ylated and gives rise to o-aminophenol (113). After injection into the rabbit it is excreted as the glucuronide of N-methyl-p-aminophenol, $HO \cdot C_6H_4 \cdot NH \cdot CH_3$ (113a).

Nitro-compounds. The reduction of the nitro-group to the amino-group is a frequent, though by no means invariable, change to which nitro-compounds are subjected in the organism. Nitrobenzene, $C_6H_5 \cdot NO_2$, undergoes both oxidation and reduction in man and the rabbit, giving rise to p-aminophenol, $HO \cdot C_6H_4 \cdot NH_2$, (158). This compound is also formed in the body from p-nitrophenol, $HO \cdot C_6H_4 \cdot NO_2$, and aniline (158). The mono-nitrotoluenes are oxidized to the corresponding nitrobenzoic acids (122, 124). It was Jaffé (124) who first drew attention to the fact that o-nitrobenzoic acid, in contrast to the m- and p-isomers, is not excreted in conjugation with glycine. He also observed that o-nitrotoluene gives rise to a derivative of o-nitrobenzyl alcohol. On hydrolysis, this derivative yields an acid which, from Jaffé's account of its properties, is almost certainly glucuronic acid.

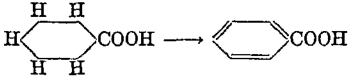
The nitrophenols tend to undergo reduction in the organism to aminophenols, and it has been suggested that their stimulatory effect on metabolism is related to this fact (94). Meyer (158) found that m- and p-nitrophenols are reduced in the rabbit to the corresponding aminophenols, but he was unable to find evidence of the occurrence of such a reduction in the case of o-nitrophenol. In the animal body, 2, 4-dinitrophenol is converted to 2-amino-4-nitrophenol and 2-nitro-4-aminophenol (89), and the nitro-group in position 2 of 2,4,6-trinitrophenol (picric acid) is said to undergo reduction, thereby forming picramic acid (276). Following the administration of the mono-nitrophenols, there is an increased ethereal sulphate excretion (16, 93, 283).

In man and the dog, the mono-nitrobenzaldehydes, $NO_2 \cdot C_6H_4 \cdot CHO$, are oxidized to the corresponding nitrobenzoic acids (259, 46, 237, 114) and here again it has been noted that whereas the m- and p-acids are excreted in conjugation with glycine, this does not occur in the case of o-nitrobenzoic acid. In the rabbit m-nitrobenzaldehyde undergoes oxidation, reduction and acetylation, and is excreted as m-acetylaminobenzoic acid, $CH_3 \cdot CO \cdot NH \cdot C_6H_4 \cdot COOH$ (46). The mono-nitrophenylacetic acids, $NO_2 \cdot C_6H_4 \cdot CH_2 \cdot COOH$, tend to be excreted unchanged by man and the rabbit; some conjugation occurs in the case of the m- and p-isomers in the dog (236, 39, 169).

Sulphonic acids. Such information as is available on the subject indicates that the sulphonic acid group, when attached to the aromatic nucleus, does not undergo modification in the body (221, 16, 136).

The increased excretion of ethereal sulphates which follows the administration of phenol and guaiacol does not take place when the sulphonic acid derivatives of these compounds are fed (16, 136).

Cyclohexane derivatives. The changes which foreign compounds of the hydroaromatic series undergo in the organism are of particular interest, in view of the physiological importance of a number of compounds which possess condensed carbocyclic structures of the reduced type. The fate of a number of cyclohexane derivatives in the body has recently been studied by Bernhard (23). He has shown, as also has Friedmann (73), that when hexahydrobenzoic acid, $C_6H_{11} \cdot COOH$, is administered to the dog, it is converted to benzoic acid:



He has also found that hexahydrohippuric acid, $C_6H_{11} \cdot CO \cdot NH \cdot CH_2 \cdot COOH$, hexahydrophenylpropionic acid, $C_6H_{11} \cdot CH_2 \cdot CH_2 \cdot COOH$, and the N-methyl and N-dimethyl derivatives of the amide of hexahydrobenzoic acid, $C_6H_{11} \cdot CO \cdot NH_2$, undergo dehydrogenation and conversion to benzoic acid in the dog. When hexahydrophenylacetic acid, $C_6H_{11} \cdot CH_2 \cdot COOH$, is administered, however, no formation of phenylacetic acid can be detected. When the hydroxy, methyl, o- and p-dimethyl, and amino derivatives of cyclohexane, C_6H_{12}, are administered, they are apparently destroyed or stored. Bernhard has also investigated the fate of the hexahydrotoluic acids, $CH_3 \cdot C_6H_{10} \cdot COOH$, and has shown that although hexahydro-m-toluic acid is dehydrogenated to yield m-toluic acid, $CH_3 \cdot C_6H_4 \cdot COOH$, dehydrogenation is only partial in the case of the p-derivative, and apparently does not occur in the case of either the cis- or trans- form of hexahydro-o-toluic acid. From these and other findings, it appears that in the organism of the dog marked dehydrogenation of cyclohexane derivatives occurs when there is a free carboxyl group substituted directly in the ring, or when such a group can be formed. Other substituent groups can also influence dehydrogenation, for example, the o-substituted methyl group exerts an inhibitory influence.

SOURCE OF THE SUBSTANCES EXCRETED IN CONJUGATION WITH CARBOCYCLIC COMPOUNDS. *Glycine.* It is known that the amount of glycine available in the body for the detoxication of benzoic acid is in excess of that present in the diet. The origin of this additional glycine is uncertain. Some workers (154, 218) have concluded that it is derived from "extra

destroyed" protein, and not from protein which would be metabolised were no benzoic acid given. It is now generally accepted, however, that the formation of hippuric acid in man and animals can involve a synthesis of glycine from nitrogen which would normally be excreted as urea (280, 150, 147, 242, 260). Experiments with both growing (51, 85, 86) and adult (87) animals have revealed that the rate at which the organism can produce glycine for detoxicating purposes is distinctly limited, and in a fasting man it is this factor which eventually limits the output of hippuric acid when increasing doses of sodium benzoate are ingested (205). Griffith and Lewis found that the administration of glycine (87), or protein digests rich in glycine (88), to rabbits receiving sodium benzoate increases the rate of excretion of hippuric acid. A number of other amino acids and other substances which have been tested fail to produce this effect. It is not due to a stimulatory influence of glycine on the excretory function of the kidneys, on the absorption of benzoate from the intestine, or on general metabolism. Griffith and Lewis (87) have suggested that this increased rate of excretion of hippuric acid is due to a greater rate of synthesis of this compound resulting from the presence of increased amounts of preformed glycine in the organism. It has also been observed that when glycine is fed to a dog (206) or a rabbit (271) receiving phenylacetic acid, the rate of excretion of phenaceturic acid is increased.

Glutamine. Under normal conditions a man can convert considerable amounts of phenylacetic acid to phenylacetylglutamine (239, 7), e.g., about 95 per cent of a dose of 10 grams of phenylacetic acid is excreted in this conjugated form (7). Shiple and Sherwin (242) have shown that glutamine can be synthesized by man for detoxicating purposes at the expense of urea nitrogen.

Ornithine. A fowl on a non-nitrogenous diet can apparently synthesize ornithine for the detoxication of benzoic acid, and this results in a decreased excretion of uric acid. The output of ornithuric acid by the fowl is increased by the administration of arginine (50).

Cysteine. Before the recent work of Stekol (250) on the determination of mercapturic acids in urine, anyone wishing to make a quantitative study of the excretion of p-bromophenylmercapturic acid, for example, was obliged either to isolate and weigh the acid or to assume that increases in the neutral sulphur of the urine after bromobenzene administration are due to mercapturic acid. It is well known that the methods available for the isolation of mercapturic acids are by no means quantitative, and in certain instances their use has yielded

misleading results (*c.f.* 1, 2, 151). On the other hand, certain workers (8, 279, 250, 252) have questioned the validity of the assumption that mercapturic acid formation is alone responsible for the increases in the neutral sulphur of the urine after giving compounds of the type of bromobenzene to animals. This assumption has been criticised (252) on the ground that certain sulphur compounds on ingestion may cause increases in the neutral sulphur of the urine which are independent of their effect on mercapturic acid formation. In the light of these considerations it is not surprising that the literature reveals some differences of opinion on the subject of the formation of mercapturic acids *in vivo*. Although early investigators (266, 133, 170) emphasized the importance of exogenous cystine in the synthesis of mercapturic acids, it now appears that the animal can use tissue sulphur for this purpose, for mercapturic acid formation has been shown to occur in various fasting animals, including the rabbit (185), cat (275), and the growing (248) and adult dog (3). The work of White and Lewis (279) on the influence of l-cystine and dl-methionine on the sulphur excretion of dogs receiving bromobenzene on a low sulphur diet, first suggested that these two amino acids behave similarly in the detoxication of bromobenzene, and it is now known that under the above conditions they both bring about an increased mercapturic acid formation (252). The growth of young rats on a basal diet in which the level of cystine and methionine is just sufficient for good growth can be inhibited by giving bromobenzene (278). The most probable explanation of this effect is that the detoxication of bromobenzene takes precedence over growth requirements under these conditions, for resumption of growth can be induced either by stopping the administration of bromobenzene (278, 91) or by the addition of cystine or methionine to the basal diet (278). Similar results have been obtained by Stekol (256). Stekol, who has made a detailed study of mercapturic acid synthesis in animals (250–258), has been led by his findings to suggest (255, 257) that the extent of mercapturic acid formation is determined by the sulphur of the diet only in so far as this influences the nutritional state of the animal.

Sulphuric acid. There has been some disagreement among various workers concerning the source of the sulphur involved in the formation of ethereal sulphates in the organism. Muldoon *et alia* (170) considered that ethereal sulphates are formed as a result of endogenous metabolism and not at the expense of inorganic sulphate. In experiments with a pig reduced to a state of endogenous nitrogen metabolism, these workers found (240) that the feeding of inorganic sulphate to-

gether with bromobenzene, phenol, or p-chlorophenol resulted in no increase in the excretion of ethereal sulphates. It has since been found that the pig does not readily form ethereal sulphates (49, 251) and it seems probable that it would be difficult to demonstrate a combination of phenolic compounds with inorganic sulphate in this animal. Sherwin and his co-workers (219, 220) obtained evidence that mercapturic acids can be oxidized in the organism, and suggested (240) that ethereal sulphates arise partly as a result of the oxidation of mercapturic acids formed from phenols. Other workers (38, 145) have failed to find evidence of the conversion of mercapturic acids to ethereal sulphates in the organism. Because of this, and from structural considerations, Hele (101, 49) has criticised the view that mercapturic acid formation is a stage in ethereal sulphate production and has suggested that König's (142) work would make it seem more probable that oxidation of mercapturic acids in the body would lead to sulphonic acid formation. Hele found that administered sodium sulphate is utilized by the dog for the formation of ethereal sulphates when guaiacol carbonate (100), phenol or indole (102) are given, and suggested (102) that "in every case ethereal sulphate, whether arising endogenously or exogenously, is formed by the union of the sulphate ion with phenolic compounds."

Acetic acid. Quantitative studies (107, 97) on the acetylation of p-aminobenzoic acid in the rabbit have shown that the excretion of p-acetylaminobenzoic acid is markedly increased by the administration of a variety of substances, including sodium acetate, pyruvic acid, and ethyl acetoacetate. Furthermore, the injection of insulin also has this effect (96). The acetylation of sulphanilamide by tissues *in vitro* has been studied by Klein and Harris (135). They showed that the acetylation of this compound can occur in slices of rabbit and human liver, and that, normally, the factor limiting the reaction under these conditions is the rate of acetate production by the tissue. Addition of acetate increases the amount of conjugation as also does the addition of substances which give rise to acetate in the tissue. The decreased acetylation which is observed, in the absence of added acetate, under anaerobic conditions or in the presence of low concentrations of arsenious oxide or iodoacetamide, is probably mainly due to an inhibition of acetate formation. Klein and Harris (135) suggest the following scheme for the acetylation of sulphanilamide by rabbit liver:

$$\begin{tikzcd}[column sep=small] \text{Lactate} \arrow[r] & \text{Pyruvate} \arrow[r] \arrow[d] & \text{Acetate} & + & \text{Sulphanilamide} \arrow[d] \\ & \text{Acetaldehyde} \arrow[ur] & & & \text{Acetylsulphanilamide} \end{tikzcd}$$

In the light of theories (274, 139, 137) concerning the rôle of acetylation in the synthesis of amino acids in the animal body, Stekol (258) has suggested that mercapturic acid formation may be the result of an interaction of ammonia, pyruvic acid, and an α-keto-β-thiopropionic acid derivative, rather than the result of direct acetylation of a cysteine derivative.

Glucuronic acid. Fischer and Piloty (68) advanced the theory that conjugated glucuronic acids arise in the organism as a result of a combination of a hydroxy-compound with glucose to yield a glucoside which undergoes oxidation to the corresponding glucuronic acid derivative. Although the reported conversion in the body of certain glucosides to their corresponding glucuronides (110, 111, 92) may be interpreted in favour of this theory, the possible hydrolysis of the glucoside followed by the conjugation of the hydroxy-compound with glucuronic acid has to be taken into consideration. Support for this possibility has come from the finding (198) that phenyl-α- and β-glucosides are hydrolysed when administered orally or subcutaneously to the rabbit,. and behave like the equivalent amount of phenol with respect to the amount of ethereal sulphate to which they give rise. The production of glucuronic acid derivatives of borneol or benzoic acid by the fasting depancreatised dog occurs as readily as in the normal dog and results in a decrease in the output of urinary sugar which corresponds to the amount of glucuronic acid excreted (201). When insulin is injected into normal dogs receiving sodium benzoate, the production of glucuronic acid is increased (210). Quick's observations have led him to suggest (202) that glucuronic acid can be produced by the animal both from carbohydrate and from protein. Miller *et alia* (160, 161) prefer to take the view that glucuronic acid liberated by the digestion of mucin is used for detoxication purposes. The findings of Ambrose and Sherwin (9) do not support this view, and they believe that the glucuronic acid used in detoxication is mainly synthesised from glycogenic substances. It has been noted that the administration of certain drugs to pentosuric subjects leads to an increased excretion of l-xyloketose, and the suggestion (64) that this is related to the formation of glucuronic acid in response to these drugs has received support from the finding that the ingestion of d-glucuronic acid by these individuals also increases the excretion of pentose (65). The feeding of mucin has been shown to have no significant effect on the excretion of l-xyloketose (65).

Site of detoxication. Information concerning the site of detoxication has been mainly derived from observations on the extent to

which the detoxication of a given compound occurs in: *a*, animals suffering from injury, exclusion or extirpation of an organ under investigation; *b*, perfusion of isolated organs; *c*, experiments with tissue slices or ground tissues. While there is still much uncertainty concerning the extent to which any one tissue is responsible for the detoxication of a given compound, it has become generally accepted that the rôle of the liver in detoxication is a major one. The kidney may also play an important part in certain detoxication reactions. Thus it appears, for example, that in the dog the kidney is the sole site of synthesis of hippuric acid. This follows from the work of Bunge and Schmiedeberg (36). Although this work has been criticized (134), it has been supported by the findings of other investigators (140, 10, 244, 207). It is interesting to note that in the dog the synthesis of phenaceturic acid can occur elsewhere than in the kidney, for phenaceturic acid has been isolated from the blood of a nephrectomized dog following the injection of phenylacetic acid (207). The oxidation of phenyl derivatives of fatty acids has been found to take place in the kidney of the dog. Snapper and Grünbaum (243) showed that the perfusion of dog kidney with blood containing glycine and phenylpropionic acid or phenylvaleric acid results in the formation of hippuric acid, whereas phenylbutyric acid gives rise to phenylaceturic acid. Although there is evidence that the kidneys of certain animals, other than the dog, are capable of bringing about the synthesis of hippuric acid (244), the kidney is not invariably the site of formation of this compound. Hippuric acid synthesis has been observed in nephrectomized rabbits (226, 207) and also as a result of the perfusion of rabbit liver with blood containing sodium benzoate (76). Quick (207) attributes this synthesis to an enzyme, hippuricese, and has concluded that whereas this enzyme is confined to the kidney in the dog, this is not so in the rabbit and in man. Quick (205, 211, 214) has shown that the rate of excretion of hippuric acid by normal persons after the ingestion of sodium benzoate is approximately constant, and that the rate is lowered in the case of persons suffering from certain types of hepatic disease. He regards this reduced rate of excretion of hippuric acid as being due primarily to a decrease in the ability of the liver to synthesise glycine and partly to injury to the enzyme system which brings about the conjugation of benzoic acid with glycine. On the basis of these considerations Quick has developed a liver function test, the clinical value of which he has studied in detail (211, 214). Other workers have confirmed the finding that there is a decreased output of hippuric acid in certain pathological conditions of the liver

produced by disease (272, 246, 141) or by poisoning with chemical agents (131, 6, 270).

There is a considerable amount of evidence which indicates that the formation of ethereal sulphates and glucuronides takes place in the liver (12, 41, 62, 63, 190, 186). Hemingway, Pryde and Williams (105) have recently studied the question of the site of glucuronic acid conjugation by applying a perfusion technique to various combinations of dog tissues. In experiments with a pump-lung-kidney preparation of the type described by Hemingway (103, 104) they showed that the isolated kidney could excrete glucuronides but could not form them. When this preparation was used in conjunction with a liver perfused in a manner similar to that described by Bauer *et alia* (11), it was found that the addition of phenol, chloral hydrate, camphor or borneol to the perfusing blood resulted in the presence of increased amounts of glucuronide in the urine. Other organs (spleen, hind-limb) were perfused with blood containing phenol, but only in those preparations in which the liver was perfused was evidence of glucuronide synthesis obtained. The importance of the liver in the formation of glucuronides is further emphasized by the decreased excretion of these compounds which has been observed in cases of hepatic disease (26, 173, 174) and poisoning of the liver by chemical agents (26, 193, 172). Recent studies by Fishman (69, 70) on β-glucuronidase have revealed that this enzyme is present in various organs of the dog, and that the amounts present increase after the feeding of large amounts of borneol. If, as seems possible, β-glucuronidase possesses synthetic activity, such a finding would suggest that the liver is not the only tissue involved in glucuronide formation. Lipschitz and Büding (149) have observed the formation of glucuronide in the presence of borneol in liver slices, and to a lesser extent in kidney slices.

The perfusion of dog liver with blood containing α-aminophenylacetic acid has been shown (181) to result in the conversion of this compound to its acetylamino derivative. Muenzen *et alia* (167) have concluded from their investigations that the acetylation of foreign compounds is probably confined to the liver. In their studies on the acetylation of sulphanilamide by tissues *in vitro*, Klein and Harris (135) were able to demonstrate the occurrence of this reaction in experiments in which they employed intact liver cells.

As the foregoing examples show, much evidence has accumulated which indicates that the liver is able to bring about the synthesis of conjugated derivatives of a variety of foreign compounds. When

these compounds are absorbed from the gastro-intestinal tract, it seems probable that the liver, by virtue of its position, plays a major part in their detoxication. With the exception of the kidney, little is known at present concerning whether or not tissues other than the liver are capable of participating in the detoxication of carbocyclic compounds.

REFERENCES

(1) Abderhalden, E. and E. Wertheimer. Pflüger's Arch. **207**: 215, 1925.
(2) Abderhalden, E. and E. Wertheimer. Pflüger's Arch. **209**: 611, 1925.
(3) Abderhalden, E. and E. Wertheimer. Ztschr. physiol. Chem. **198**: 18, 1931.
(4) Abderhalden, E. and E. Wertheimer. Ztschr. physiol. Chem. **201**: 267, 1931.
(5) Adams, D. R. Brit. J. Ophthal. **14**: 49, 1930.
(6) Adlersberg, D. and H. Minibeck. Ztschr. ges. exper. Med. **98**: 185, 1936.
(7) Ambrose, A. M., F. W. Power and C. P. Sherwin. J. Biol. Chem. **101**: 669, 1933.
(8) Ambrose, A. M. and C. P. Sherwin. Ann. Rev. Biochem. **2**: 377, 1933.
(9) Ambrose, A. M. and C. P. Sherwin. J. Biol. Chem. **105**: iv, 1934.
(10) Bashford, E. and W. Cramer. Ztschr. physiol. Chem. **35**: 324, 1902.
(11) Bauer, W., H. H. Dale, L. T. Poulsson and D. W. Richards. J. Physiol. **74**: 343, 1932.
(12) Baumann, E. Pflüger's Arch. **13**: 285, 1876.
(13) Baumann, E. Ztschr. physiol. Chem. **2**: 335, 1878–9.
(14) Baumann, E. Ztschr. physiol. Chem. **7**: 282, 1882–3.
(15) Baumann, E. Ztschr. physiol. Chem. **8**: 190, 1883.
(16) Baumann, E. and E. Herter. Ztschr. physiol. Chem. **1**: 244, 1877–8.
(17) Baumann, E. and C. Preusse. Ber. deutsch. chem. Gesellsch. **12**: 806, 1879.
(18) Baumann, E. and C. Preusse. Ztschr. physiol. Chem. **3**: 156, 1879.
(19) Baumann, E. and C. Preusse. Ztschr. physiol. Chem. **5**: 309, 1881.
(21) Baumann, E. and P. Schmitz. Ztschr. physiol. Chem. **20**: 586, 1895.
(22) Bergel, P. and R. Pschorr. Ztschr. physiol. Chem. **38**: 17, 1903.
(23) Bernhard, K. Ztschr. physiol. Chem. **248**: 256, 1937.
(24) Bertagnini, C. Ann. der Chemie **97**: 248, 1856.
(25) Boeseken, J. and G. Sloof. Proc. Acad. Sci. Amsterdam. **32**: 1043, 1929.
(26) Boku, S. and T. Kin. J. Chosen Med. Assn. **21**: 67, 1931 (*In* Chem. Abstr. **26**: 3834, 1932).
(27) Bouchard and Charrin. Compt. Rend. Soc. de Biol. **8**: 614, 1886.
(28) Bourne, M. C. and L. Young. Chem. and Ind. **52**: 271, 1933.
(29) Bourne, M. C. and L. Young. Chem. and Ind. **52**: 1056, 1933.
(30) Bourne, M. C. and L. Young. Biochem. J. **28**: 803, 1934.
(31) Boyland, E. and A. A. Levi. Biochem. J. **29**: 2678, 1935.
(32) Boyland, E. and A. A. Levi. Biochem. J. **30**: 728, 1936.
(33) Boyland, E. and A. A. Levi. Biochem. J. **30**: 1225, 1936.
(34) Brakefield, J. L. and C. L. A. Schmidt. J. Biol. Chem. **67**: 523, 1926.
(35) Brieger, L. Ztschr. physiol. Chem. **2**: 241, 1878–9.

(36) BUNGE, G. AND O. SCHMIEDEBERG. Arch. exper. Path. u. Pharmakol. 6: 233, 1876–7.
(37) CALLOW, E. H. AND T. S. HELE. Biochem. J. 20: 598, 1926.
(38) CALLOW, E. H. AND T. S. HELE. Biochem. J. 21: 606, 1927.
(39) CERECEDO, L. R. AND C. P. SHERWIN. J. Biol. Chem. 58: 215, 1923–4.
(40) CERECEDO, L. R. AND C. P. SHERWIN. J. Biol. Chem. 62: 217, 1924–5.
(41) CHRISTIANI, A. AND E. BAUMANN. Ztschr. physiol. Chem. 2: 350, 1878–9.
(42) COHEN, S. L. AND G. F. MARRIAN. Biochem. J. 29: 1577, 1935.
(43) COHEN, S. L. AND G. F. MARRIAN. Biochem. J. 30: 57, 1936.
(44) COHEN, S. L., G. F. MARRIAN AND M. WATSON. Lancet 1: 674, 1935.
(45) ÇOHN, R. Ztschr. physiol. Chem. 14: 203, 1890.
(46) COHN, R. Ztschr. physiol. Chem. 17: 274, 1893.
(47) COOMBS, H. I. Biochem. J. 21: 623, 1927.
(48) COOMBS, H. I. AND T. S. HELE. Biochem. J. 20: 606, 1926.
(49) COOMBS, H. I. AND T. S. HELE. Biochem. J. 21: 611, 1927.
(50) CROWDLE, J. H. AND C. P. SHERWIN. J. Biol. Chem. 55: 365, 1923.
(51) CSONKA, F. A. J. Biol. Chem. 60: 545, 1924.
(52) CURCI, A. Jahresb. f. Tierchemie. 24: 100, 1894.
(53) DAKIN, H. D. J. Biol. Chem. 5: 173, 1908–9.
(54) DAKIN, H. D. J. Biol. Chem. 6: 221, 1909.
(55) DAKIN, H. D. J. Biol. Chem. 7: 103, 1909–10.
(56) DAKIN, H. D. Oxidations and reductions in the animal body. London, 1922.
(57) DIACK, S. L. AND H. B. LEWIS. J. Biol. Chem. 77: 89, 1928.
(58) DIXON, M. AND C. LUTWAK-MANN. Biochem. J. 31: 1347, 1937.
(59) DRUMMOND, J. C. AND I. L. FINAR. Biochem. J. 32: 79, 1938.
(60) DUBIN, H. J. Biol. Chem. 26: 69, 1916.
(61) EDLEFSEN, G. Arch. exper. Path. u. Pharmakol. 52: 429, 1905.
(62) EMBDEN, G. Beitr. chem. Physiol. u. Path. 2: 591, 1903.
(63) EMBDEN, G. AND K. GLAESSNER. Beitr. chem. Physiol. u. Path. 1: 310, 1902.
(64) ENKLEWITZ, M. AND M. LASKER. Am. J. Med. Sci. 186: 539, 1933.
(65) ENKLEWITZ, M. AND M. LASKER. J. Biol. Chem. 110: 443, 1935.
(66) EWINS, A. J. AND P. P. LAIDLAW. J. Physiol. 41: 78, 1910.
(67) EWINS, A. J. AND P. P. LAIDLAW. Biochem. J. 7: 18, 1913.
(68) FISCHER, E. AND O. PILOTY. Ber. deutsch. chem. Gesellsch. 24: 521, 1891.
(69) FISHMAN, W. H. J. Biol. Chem. 123: xxxvi, 1938.
(70) FISHMAN, W. H. Private communication to author. 1938.
(71) FOLIN, O. AND W. DENIS. J. Biol. Chem. 22: 309, 1915.
(72) FRIEDMANN, E. Biochem. Ztschr. 35: 40, 1911.
(73) FRIEDMANN, E. Biochem. Ztschr. 35: 49, 1911.
(74) FRIEDMANN, E. Biochem. Ztschr. 242: 288, 1931.
(75) FRIEDMANN, E. AND H. MAI. Biochem. Ztschr. 242: 282, 1931.
(76) FRIEDMANN, E. AND H. TACHAU. Biochem. Ztschr. 35: 88, 1911.
(77) FRIEDMANN, E. AND W. TURK. Biochem. Ztschr. 55: 425, 1913.
(78) FUCHS, D. AND A. v. SOÓS. Ztschr. physiol. Chem. 98: 1, 1916–7.
(79) FULLER, A. T. Lancet 1: 194, 1937.
(80) GLEDITSCH, A. AND H. MOELLER. Ann. der Chemie 250: 376, 1889.

(81) Go, J. Jap. J. Exper. Med. **2**: 77, 1927.
(82) GOEBEL, W. F. J. Biol. Chem. **122**: 649, 1937–8.
(83) GOEBEL, W. F. AND F. H. BABERS. J. Biol. Chem. **111**: 347, 1935.
(84) GRIFFITH, W. H. J. Biol. Chem. **69**: 197, 1926.
(85) GRIFFITH, W. H. J. Biol. Chem. **82**: 415, 1929.
(86) GRIFFITH, W. H. J. Biol. Chem. **85**: 751, 1929–30.
(87) GRIFFITH, W. H. AND H. B. LEWIS. J. Biol. Chem. **57**: 1, 1923.
(88) GRIFFITH, W. H. AND H. B. LEWIS. J. Biol. Chem. **57**: 697, 1923.
(89) GUERBET, M. AND M. ANDRE. Ann. physiol. physicochim. biol. **8**: 117, 1932. (*Cited by* HARROW, B. and C. P. SHERWIN (98).)
(90) GUGGENHEIM, M. AND W. LOEFFLER. Biochem. Ztschr. **72**: 325, 1915–6.
(91) HALEY, F. L. AND G. S. SAMUELSON. J. Biol. Chem. **119**: 383, 1937.
(92) HÄMÄLÄINEN, J. Skand. Arch. f. Physiol. **30**: 187, 196, 1913.
(93) HAMMERBACHER, F. Pflüger's Arch. **33**: 94, 1883.
(94) HANDOVSKY, H., H. CASIER AND C. SCHEPENS. Compt. Rend. Soc. Biol. **115**: 1727, 1934.
(95) HANZLIK, P. J. J. Pharmacol. and Exper. Therap. **10**: 461, 1917–8.
(96) HARROW, B., A. MAZUR AND C. P. SHERWIN. J. Biol. Chem. **102**: 35, 1933.
(97) HARROW, B., F. W. POWER AND C. P. SHERWIN. Proc. Soc. Exper. Biol. and Med. **24**: 422, 1926–7.
(98) HARROW, B. AND C. P. SHERWIN. Ann. Rev. Biochem. **4**: 263, 1935.
(99) HEFFTER, A. Ergebn. Physiol. **4**: 184, 1905.
(100) HELE, T. S. Biochem. J. **18**: 110, 1924.
(101) HELE, T. S. Biochem. J. **18**: 586, 1924.
(102) HELE, T. S. Biochem. J. **25**: 1736, 1931.
(103) HEMINGWAY, A. J. Physiol. **71**: 201, 1931.
(104) HEMINGWAY, A. J. Physiol. **77**: 14 p, 1933.
(105) HEMINGWAY, A., J. PRYDE AND R. T. WILLIAMS. Biochem. J. **28**: 136, 1934.
(106) HENSCHEN, C. Arch. klin. Chir. **189**: 19, 1937.
(107) HENSEL, M. Ztschr. physiol. Chem. **93**: 401, 1915.
(108) HERMANS, L. Ztschr. physiol. Chem. **85**: 233, 1913.
(109) HILDEBRANDT, H. Beitr. chem. Physiol. u. Path. **3**: 365, 1903.
(110) HILDEBRANDT, H. Beitr. chem. Physiol. u. Path. **7**: 438, 1905.
(111) HILDEBRANDT, H. Biochem. Ztschr. **21**: 1, 1909.
(112) HOLMES, E. G. J. Pharmacol. and Exper. Therap. **26**: 297, 1925–6.
(113) HORN, F. Ztschr. physiol. Chem. **238**: 84, 1936.
(113a) HORN, F. Ztschr. physiol. Chem. **242**: 23, 1936.
(114) HOSODA, T. J. Biochem. (Japan) **6**: 171, 1926.
(115) HOTCHKISS, R. D. AND W. F. GOEBEL. J. Biol. Chem. **115**: 285, 1936.
(116) HUEPER, W. C., F. H. WILEY AND H. D. WOLFE. J. Ind. Hyg. and Toxicol. **20**: 46, 1938.
(117) IGERSHEIMER, J. AND L. RUBIN. Arch. f. Ophthal. **74**: 467, 1910.
(118) IMAI, T. Acta Schol. Med. Univ. Kioto **6**: 415, 1924.
(119) INAGAKI, S. Ztschr. physiol. Chem. **214**: 25, 1933.
(120) ING, H. R., M. C. BOURNE AND L. YOUNG. Biochem. J. **28**: 809, 1934.
(121) JACOBSEN, O. Ber. deutsch. chem. Gesellsch. **12**: 1512, 1879.
(122) JAFFÉ, M. Ber. deutsch. chem. Gesellsch. **7**: 1673, 1874.
(123) JAFFÉ, M. Ber. deutsch. chem. Gesellsch. **10**: 1925, 1877.

(124) Jaffé, M. Ztschr. physiol. Chem. 2: 47, 1878.
(125) Jaffé, M. Ber. deutsch. chem. Gesellsch. 12: 1092, 1879.
(126) *Jaffé, M. Ztschr. physiol. Chem. 62: 58, 1909.*
(127) Jaffé, M. and R. Cohn. Ber. deutsch. chem. Gesellsch. 20: 2311, 1887.
(128) Jaffé, M. and R. Cohn. Ber. deutsch. chem. Gesellsch. 21: 3461, 1888.
(129) Jaffé, M. and P. Hilbert. Ztschr. physiol. Chem. 12: 295, 1888.
(130) Kammer, A. G., N. Isenberg and M. E. Berg. J. A. M. A. 111: 1452, 1938.
(131) Kanzaki, I. Sei-i-Kwai Med. J. 52: 85, 1933. (*In* Chem. Abstr. 28: 2402, 1934.)
(132) Kapeller-Adler, R. and J. Kraal. Biochem. Ztschr. 235: 394, 1931.
(133) Kapfhammer, J. Ztschr. physiol. Chem. 116: 302, 1921.
(134) Kingsbury, F. B. and E. T. Bell. J. Biol. Chem. 21: 297, 1915.
(135) Klein, J. R. and J. S. Harris. J. Biol. Chem. 124: 613, 1938.
(136) Knapp, T. and F. Suter. Arch. exper. Path. u. Pharmakol. 50: 332, 1903.
(137) Knoop, F. and J. G. Blanco. Ztschr. physiol. Chem. 146: 267, 1925.
(138) Knoop, F. and M. Gehrke. Ztschr. physiol. Chem. 146: 63, 1925.
(139) Knoop, F. and E. Kertess. Ztschr. physiol. Chem. 71: 252, 1911.
(140) Kochs, W. Pflüger's Arch. 20: 64, 1879.
(141) Kohlstaedt, K. G. and O. M. Helmer. Am. J. Dig. Dis. and Nutr. 3: *501, 1936.*
(142) König, G. Ztschr. physiol. Chem. 16: 525, 1892.
(143) Külz, E. Pflüger's Arch. 30: 484, 1883.
(144) Külz, E. Ztschr. f. Biol. 27: 247, 1890.
(145) Lawrie, N. R. Biochem. J. 25: 1037, 1931.
(146) Lesnik, M. Arch. exper. Path. u. Pharmakol. 24: 167, 1888.
(147) Lewis, H. B. J. Biol. Chem. 18: 225, 1914.
(148) Lewis, H. B. Physiol. Rev. 4: 394, 1924.
(149) Lipschitz, W. and E. Büding. Kongressbericht II des XVI Internat. Physiol. Kongr., 120, 1938.
(150) McCollum, E. V. and W. R. Hoagland. J. Biol. Chem. 16: 321, 1913.
(151) McGuinn, A. F. and C. P. Sherwin. Proc. Soc. Exper. Biol. and Med. 30: 1115, 1932–3.
(152) Macht, D. I. J. Pharmacol. and Exper. Therap. 11: 263, 1918.
(153) Magnus-Levy, A. Biochem. Ztschr. 6: 502, 1907.
(154) Magnus-Levy, A. Biochem. Ztschr. 6: 523, 1907.
(155) Marshall, E. K., W. C. Cutting and K. Emerson. Science 86: 202, 1937.
(156) Masamune, H. J. Biochem. (Japan) 20: 311, 1934.
(157) Mayer, P. and C. Neuberg. Ztschr. physiol. Chem. 29: 256, 1900.
(158) Meyer, E. Ztschr. physiol. Chem. 46: 497, 1905.
(159) Michel, H. O., F. Bernheim and M. L. C. Bernheim. J. Pharmacol. and Exper. Therap. 61: 321, 1937.
(160) Miller, C. O., F. G. Brazda and E. C. Elliot. Proc. Soc. Exper. Biol. and Med. 30: 633, 1932–3.
(161) Miller, C. O. and J. A. Conner. Proc. Soc. Exper. Biol. and Med. 30: 630, 1932–3.
(162) Miriam, S. R., J. T. Wolf and C. P. Sherwin. J. Biol. Chem. 71: 249, 1927.
(163) Miriam, S. R., J. T. Wolf and C. P. Sherwin. J. Biol. Chem. 71: 695, 1927.

(164) Mori, Y. J. Biol. Chem. 35: 341, 1918.
(165) Mörner, K. A. H. Ztschr. physiol. Chem. 13: 12, 1889.
(166) Mosso, U. Arch. exper. Path. u. Pharmakol. 26: 267, 1890.
(167) Muenzen, J. B., L. R. Cerecedo and C. P. Sherwin. J. Biol. Chem. 63: xvi, 1925.
(168) Muenzen, J. B., L. R. Cerecedo and C. P. Sherwin. J. Biol. Chem. 67: 469, 1925.
(169) Muenzen, J. B., L. R. Cerecedo and C. P. Sherwin. J. Biol. Chem. 68: 503, 1926.
(170) Muldoon, J. A., G. J. Shiple and C. P. Sherwin. J. Biol. Chem. 59: 675, 1924.
(171) Munk, I. Pflüger's Arch. 12: 142, 1876.
(172) Nasarijanz, B. A. Ber. Ukrain. Biochem. Inst. 3: 159, 1928. (*In* Chem. Abstr. 30: 5295, 1936.)
(173) Nasarijanz, B. A. Ztschr. ges. exper. Med. 30: 11, 1931.
(174) Nasarijanz, B. A. Schweiz. med. Wchnschr. 64: 1090, 1934.
(175) Nencki, M. Arch. Anat. u. Physiol., 399, 1870.
(176) Nencki, M. Arch. exper. Path. u. Pharmakol. 1: 420, 1873.
(177) Nencki, M. J. f. prakt. Chemie. 18: 288, 1878.
(178) Nencki, M. Ber. deutsch. chem. Gesellsch. 27: 2732, 1894.
(179) Nencki, M. and P. Giacosa. Ztschr. physiol. Chem. 4: 325, 1880.
(180) Neubauer, O. Arch. exper. Path. u. Pharmakol. 46: 133, 1901.
(181) Neubauer, O. and O. Warburg. Ztschr. physiol. Chem. 70: 1, 1910–11.
(182) Neuberg, C. and W. Niemann. Ztschr. physiol. Chem. 44: 114, 1905.
(183) Neuberg, J. Biochem. Ztschr. 145: 249, 1924.
(184) Neumaerker, H. Ztschr. physiol. Chem. 126: 203, 1923.
(185) Nishimura, K. Acta Schol. Med. Univ. Kioto 12: 73, 1929–30.
(186) Nishimura, K. Proc. Jap. Pharm. Soc. 9: 111, 1935. (*In* Chem. Abstr. 30: 3519, 1936.)
(187) Novello, N. J., S. R. Miriam and C. P. Sherwin. J. Biol. Chem. 67: 555, 1926.
(188) Odell, A. D. and G. F. Marrian. Biochem. J. 30: 1533, 1936.
(189) Parnas, J. Biochem. Ztschr. 28: 274, 1910.
(190) Pelkan, K. F. and G. H. Whipple. J. Biol. Chem. 50: 513, 1922.
(191) Penzoldt, F. Arch. exper. Path. u. Pharmakol. 21: 34, 1886.
(192) Perkin, W. H. Trans. Chem. Soc. 21: 53, 1868.
(193) Persova, E. M. Ber. Ukrain. Biochem. Inst. 4: 43, 1930. (*In* Chem. Abstr. 30: 5295, 1936.)
(194) Power, F. W. Proc. Soc. Exper. Biol. and Med. 33: 598, 1936.
(195) Preusse, C. Ztschr. physiol. Chem. 4: 209, 1880.
(196) Preusse, C. Ztschr. physiol. Chem. 5: 57, 1881.
(197) Pryde, J. and R. T. Williams. Biochem. J. 27: 1210, 1933.
(198) Pryde, J. and R. T. Williams. Biochem. J. 30: 794, 1936.
(199) Quick, A. J. J. Biol. Chem. 67: 477, 1926.
(200) Quick, A. J. J. Biol. Chem. 69: 549, 1926.
(201) Quick, A. J. J. Biol. Chem. 70: 59, 1926.
(202) Quick, A. J. J. Biol. Chem. 70: 397, 1926.
(203) Quick, A. J. J. Biol. Chem. 77: 581, 1927.

(204) QUICK, A. J. J. Biol. Chem. 80: 515, 1928.
(205) QUICK, A. J. J. Biol. Chem. 92: 65, 1931.
(206) QUICK, A. J. J. Biol. Chem. 95: 189, 1932.
(207) QUICK, A. J. J. Biol. Chem. 96: 73, 1932.
(208) QUICK, A. J. J. Biol. Chem. 96: 83, 1932.
(209) QUICK, A. J. J. Biol. Chem. 97: 403, 1932.
(210) QUICK, A. J. J. Biol. Chem. 98: 537, 1932.
(211) QUICK, A. J. Am. J. Med. Sci. 185: 630, 1933.
(212) QUICK, A. J. J. Biol. Chem. 101: 475, 1933.
(213) QUICK, A. J. Biochem. J. 28: 403, 1934.
(214) QUICK, A. J. Arch. Int. Med. 57: 544, 1936.
(215) QUICK, A. J. Ann. Rev. Biochem. 6: 291, 1937.
(216) RHODE, H. Ztschr. physiol. Chem. 124: 15, 1923.
(217) RICHTER, D. Biochem. J. 32: 1763, 1938.
(218) RINGER, A. I. J. Biol. Chem. 10: 327, 1911-2.
(219) ROSE, A. R., G. J. SHIPLE AND C. P. SHERWIN. Unpublished, cited by G. J. SHIPLE *et al.* (240).
(220) ROSE, A. R., G. J. SHIPLE AND C. P. SHERWIN. Am. J. Physiol. 69: 518, 1924.
(221) SALKOWSKI, E. Pflüger's Arch. 4: 91, 1871.
(222) SALKOWSKI, E. Ztschr. physiol. Chem. 42: 213, 1904.
(223) SALKOWSKI, E. AND C. NEUBERG. Biochem. Ztschr. 2: 307, 1907.
(224) SALKOWSKI, E. AND H. SALKOWSKI. Ber. deutsch. chem. Gesellsch. 12: 653, 1879.
(225) SALKOWSKI, E. AND H. SALKOWSKI. Ztschr. physiol. Chem. 7: 161, 1882-3.
(226) SALOMON, W. Ztschr. physiol. Chem. 3: 365, 1879.
(227) SCHACHTER, B. AND G. F. MARRIAN. Proc. Soc. Exper. Biol. and Med. 35: 222, 1936.
(228) SCHEMPP, E. Ztschr. physiol. Chem. 117: 41, 1921-2.
(229) SCHMIEDEBERG, O. Arch. exper. Path. u. Pharmakol. 8: 1, 1878.
(230) SCHMIEDEBERG, O. Arch. exper. Path. u. Pharmakol. 14: 288, 1881.
(231) SCHUBENKO, G. Jahresb. f. Tierchemie 13: 95, 1893.
(232) SCHULTZEN, O. AND B. NAUNYN. Arch. f. Anat. u. Physiol. 349, 1867.
(233) SHERWIN, C. P. J. Biol. Chem. 31: 307, 1917.
(234) SHERWIN, C. P. Physiol. Rev. 2: 238, 1922.
(235) SHERWIN, C. P. Textbook of biochemistry (HARROW AND SHERWIN). Philadelphia, 1935, p. 366.
(236) SHERWIN, C. P. AND M. HELFAND. J. Biol. Chem. 40: 17, 1919.
(237) SHERWIN, C. P. AND W. A. HYNES. J. Biol. Chem. 47: 297, 1921.
(238) SHERWIN, C. P., G. J. SHIPLE AND A. R. ROSE. J. Biol. Chem. 73: 607, 1927.
(239) SHERWIN, C. P., M. WOLF AND W. WOLF. J. Biol. Chem. 37: 113, 1919.
(240) SHIPLE, G. J., J. A. MULDOON AND C. P. SHERWIN. J. Biol. Chem. 60: 59, 1924.
(241) SHIPLE, G. J. AND C. P. SHERWIN. J. Biol. Chem. 53: 463, 1922.
(242) SHIPLE, G. J. AND C. P. SHERWIN. J. Am. Chem. Soc. 44: 618, 1922.
(243) SNAPPER, I. AND A. GRÜNBAUM. Biochem. Ztschr. 150: 12, 1924.
(244) SNAPPER, I., A. GRÜNBAUM AND J. NEUBERG. Biochem. Ztschr. 145: 40, 1924.

(245) Snapper, I., A. Grünbaum and S. Starkop. Biochem. Ztschr. **155**: 163, 1924–5.
(246) Snell, A. M. and J. E. Plunkett. Am. J. Dig. Dis. and Nutr. **2**: 716, 1936.
(247) Stekol, J. A. J. Biol. Chem. **110**: 463, 1935.
(248) Stekol, J. A. Proc. Soc. Exper. Biol. and Med. **33**: 119, 1935–6.
(249) Stekol, J. A. Proc. Soc. Exper. Biol. and Med. **33**: 170, 1935–6.
(250) Stekol, J. A. J. Biol. Chem. **113**: 279, 1936.
(251) Stekol, J. A. J. Biol. Chem. **113**: 675, 1936.
(252) Stekol, J. A. J. Biol. Chem. **117**: 147, 1937.
(253) Stekol, J. A. J. Biol. Chem. **118**: 155, 1937.
(254) Stekol, J. A. J. Biol. Chem. **121**: 87, 1937.
(255) Stekol, J. A. J. Biol. Chem. **121**: 93, 1937.
(256) Stekol, J. A. J. Biol. Chem. **122**: 55, 1937–8.
(257) Stekol, J. A. J. Biol. Chem. **122**: 333, 1937–8.
(258) Stekol, J. A. J. Biol. Chem. **124**: 129, 1938.
(259) Sieber, N. and A. Smirnow. Monatschr. f. Chemie. **8**: 88, 1887.
(260) Swanson, W. W. J. Biol. Chem. **62**: 565, 1924–5.
(261) Takahashi, M. J. Biochem. (Japan) **11**: 437, 1930.
(262) Thierfelder, H. and W. Daiber. Ztschr. physiol. Chem. **130**: 380, 1923.
(263) Thierfelder, H. and E. Klenk. Ztschr. physiol. Chem. **141**: 29, 1924.
(264) Thierfelder, H. and C. P. Sherwin. Ber. deutsch. chem. Gesellsch. **47**: 2630, 1914.
(265) Thierfelder, H. and C. P. Sherwin. Ztschr. physiol. Chem. **94**: 1, 1915.
(266) Thomas, K. and H. Straczewski. Arch. f. Anat. u. Physiol., Physiol. Abt. 249, 1919.
(267) Tisdall, F. F. J. Biol. Chem. **44**: 409, 1920.
(268) Tollens, C. Ztschr. physiol. Chem. **67**: 138, 1910.
(269) Totani, G. Ztschr. physiol. Chem. **68**: 75, 1910.
(270) Tulane, V. J., A. A. Christman and H. B. Lewis. J. Biol. Chem. **103**: 141, 1933.
(271) Tulane, V. J. and H. B. Lewis. J. Biol. Chem. **103**: 151, 1933.
(272) Vaccaro, P. F. Surg., Gynec. and Obstet. **61**: 36, 1935.
(273) Venning, E. M. and J. S. L. Browne. Proc. Soc. Exper. Biol. and Med. **34**: 792, 1936.
(274) du Vigneaud, V. and O. J. Irish. J. Biol. Chem. **122**: 349, 1937–8.
(275) Virtue, R. W. Proc. Soc. Exper. Biol. and Med. **34**: 425, 1936.
(276) Walko, K. Arch. exper. Path. u. Pharmakol. **46**: 181, 1901.
(277) Weyl, T. Ztschr. physiol. Chem. **3**: 312, 1879.
(278) White, A. and R. W. Jackson. J. Biol. Chem. **111**: 507, 1935.
(279) White, A. and H. B. Lewis. J. Biol. Chem. **98**: 607, 1932.
(280) Wiechowski, W. Beitr. chem. Physiol. u. Path. **7**: 204, 1906.
(281) Wiley, F. H. J. Biol. Chem. **123**: cxxvii, 1938.
(282) Wiley, F. H. J. Biol. Chem. **124**: 627, 1938.
(283) Williams, R. T. Biochem. J. **32**: 878, 1938.
(284) Wohler, F. and F. Frerichs. Ann. der Chemie **65**: 335, 1848.
(285) Yant, W. P., H. H. Schrenk, R. R. Sayers, A. A. Horvath and W. H. Reinhart. J. Ind. Hyg. and Toxicol. **18**: 67, 1936.

COENZYMES

C. A. BAUMANN AND F. J. STARE

Department of Biochemistry and Cancer Research Laboratory, University of Wisconsin, Madison

Most enzymes are heat-labile, non-dialyzable, proteinaceous compounds of high molecular weight, which have been produced by living cells, and which function as catalysts in specific chemical reactions. Coenzymes, like enzymes, are catalysts produced by the living cell, and are necessary in enzymic reactions, but they are compounds of lower molecular weight, heat stable, and dialyzable. Though both enzymes and coenzymes are elaborated by the living cell, they may be separated from it and studied in vitro.

The term coenzyme was introduced in 1897 by Bertrand, who applied it to calcium and to manganese salts necessary for the action of certain plant enzymes such as pectase and laccase (1). In 1904 Harden and Young found that the thermostable, dialysable fraction of boiled yeast juice greatly increased the fermentation of cell-free extracts (2, 3). *They termed their active substance the coenzyme of yeast juice.* Since the proteinaceous enzyme-complex of yeast was called "zymase," the coenzyme of yeast juice came to be called cozymase (4).

The earlier workers believed that coenzymes "activated" enzymes, and many other examples of coenzyme activity were reported, such as the activation of plant diastase by asparagin (5), the action of inorganic chlorides on pancreatic diastase (5), the action of calcium on the coagulation of casein by rennin (5). Sodium cholate and glycocholate also showed "coenzyme activity" (6). A coenzyme for the urease of the soy bean was reported (7), though pure crystalline urease does not require a coenzyme (8).

In 1918 Meyerhof made the important observation that the coenzyme of alcoholic fermentation (Harden and Young's coenzyme) occurs in muscle, animal organs, and milk, as well as in yeast (9). The most active preparations were made by extracting frog muscle with boiling water. This was the first preparation of muscle "Kochsaft," which has been used so extensively in coenzyme research. Meyerhof further observed that this "boiled juice" greatly increased the respiration of

(32, 36). The gray matter of the brain contains much more than the white matter (36). In the adrenals the concentration is highest in the cortex (36). There is considerable species variation, the blood of birds, for example, containing much more than the blood of mammals (29, 32). It has been found in invertebrate muscle (39) and in tumors (40). Small amounts of DPN are present in certain mushrooms (41), and in the seeds or root-tips of certain higher plants (27, 42, 43).

Triphosphopyridine nucleotide was discovered in red blood cells (18). It has also been isolated from them (16), from heart muscle and from yeast (44). Later work has indicated it to be very widely distributed in cells (29); and Warburg claims it to be a "ubiquitous cell constituent" (45).

Determination of diphosphopyridine nucleotide. 1. *Euler and Myrbäck* (30, 46–48). The most widely used method for the determination of diphosphopyridine nucleotide involves an artificial fermenting system in which all the necessary factors are present except the coenzyme. When DPN is added, the system ferments, and the CO_2 evolved is a measure of the amount of coenzyme present. The CO_2 is measured manometrically in a Warburg apparatus. A unit of cozymase is that amount which will liberate 1 cc. of CO_2 per hour under standard conditions. The purity of a preparation equals the units per gram of dry weight. A typical determination contains the following: 0.3 gram "apozymase,"[1] 0.1 gram glucose, 0.15 mol. phosphate, 5 mgm. hexose diphosphate, DPN solution and water to make 2 cc.

The apozymase is a washed yeast preparation containing a mixture of enzymes necessary in fermentation. It is prepared as follows: Pressed yeast is dried in air, then suspended in water and washed many times. The pasty mass is removed and dried on a glass plate. It has no action in the absence of DPN, and certain preparations are also deficient in Mg.

2. *Warburg's "Gär test"* (20). The chief criticism of the Euler-Myrbäck method is the uncertainty involved in the use of crude materials such as "apozymase." Warburg's method involves the use of purified materials. The reacting mixture contains (a) proteins A and B from yeast prepared according to Negelein (49); (b) inorganic salts Mn, Mg, NH_4; (c) substrates hexose monophosphate (Robison ester), H_3PO_4, and acetaldehyde; (d) the coenzymes diphosphopyridine nu-

[1] According to v. Euler's nomenclature the entire enzyme-complex needed for a reaction is called the "holoenzyme", i.e., holozymase. That which is left after removal of substrate and coenzyme is called the "apoenzyme", i.e., apozymase.

cleotide and adenosin triphosphate. Both are needed for a reaction. When either is present alone, the test can be used for the detection of the other.

The reaction is carried out in 0.3 per cent $NaHCO_3$ solution in a standard Warburg flask, and the gas space is filled with an inert gas containing 10 per cent CO_2. (Warburg uses argon.) The chief reaction involved is the reduction of acetaldehyde to ethyl alcohol, with a concurrent break-down of the hexose monophosphate. The over-all reaction appears to be:

$$\text{hexose monophosphate} + 2\,CH_3\,CHO + H_2O \rightarrow$$
$$CH_3\,CO\,COOH + CH_2OH_2PO_3\,CHOH\,COOH + 2\,CH_3CH_2OH$$

The production of pyruvic and phosphoglyceric acids results in an increase in the acidity of the solution causing the liberation of CO_2 from the bicarbonate, in amounts proportional to the activity of the coenzyme present. The gas is measured manometrically.

The Warburg test differs from the Euler-Myrbäck test in several particulars: the Euler-Myrbäck reaction attempts to duplicate the entire fermentation process; the Warburg reaction involves only a part of the process. In the Euler reaction the CO_2 evolved comes from the breakdown of the substrate; in the Warburg test, the CO_2 evolved is an index of the change in acidity of the mixture.

3. *Chemical determination of the pyridine nucleotides.* Both diphosphopyridine nucleotide and the triphosphopyridine nucleotide react similarly in this determination. The coenzyme as isolated (oxidized form) does not fluoresce in ultraviolet light, nor does it have an absorption band in the near ultraviolet. On the contrary, the reduced coenzyme exhibits a white fluorescence in ultraviolet light, and has a pronounced absorption band with a maximum at 340 mμ (16, 20). Thus it is possible to determine the amount of coenzyme present in a material by first reducing it, either enzymatically or by means of chemicals such as sodium hydrosulfite, and then quantitatively determining the amount of fluorescence, or the change in absorption at 340 mμ (16, 20, 50, 51).

These coenzymes can also be determined by completely reducing the pyridine ring with hydrogen in the presence of Pt. A borate buffer is used. In this reaction one molecule of coenzyme absorbs 6 atoms of hydrogen (16, 52).

Determination of triphosphopyridine nucleotide. TPN is determined by measuring the rate of oxygen absorption in a system in which it is

the limiting factor, the materials present being hexose monophosphate, dehydrogenase, yellow enzyme, and oxygen in addition to the coenzyme (16). If the other materials are present in excess, the rate of oxygen uptake is proportional to the amount of TPN present.

Preparation of diphosphopyridine nucleotide. (Method of Euler and Myrbäck (30).) Washed top or bottom yeast with a cozymase value (A Co) of 100 units is added to 4 volumes of water at 75 to 80°C. with stirring. The mass is filtered through linen, or centrifuged, and the filtrate concentrated in vacuo at 60°. The material is then dialyzed for 5 hours through parchment, 80 per cent of the coenzyme passing into the dialysate, which has a strong green fluorescence. The solution is treated with lead acetate, the precipitate filtered off, suspended in water, and decomposed with H_2S. Further purification is effected by absorbing on freshly precipitated $Al(OH)_3$ and diluting with H_3PO_4. The coenzyme can be precipitated with $HgNO_3$, picric acid, or phosphotungstic acid. The product obtained by the above procedure consists of diphosphopyridine nucleotide plus large amounts of adenine nucleotide.

For other methods of preparation see 21, 53–57 and especially 58.

Preparation of both pyridine nucleotides. (Method of Warburg (19, 20).) This method yields three coenzymes in pure form, diphosphopyridine nucleotide, triphosphopyridine nucleotide, and adenylic acid. Washed red blood cells are cytolyzed in distilled water, and the proteins precipitated with acetone. The solution is centrifuged, the liquid phase concentrated in vacuo, and treated with mercury acetate. The precipitate contains the three coenzymes. It is decomposed with H_2S, and the nucleotides precipitated with acetone. One thousand liters of red blood cells yielded 48 grams of raw nucleotides (20).

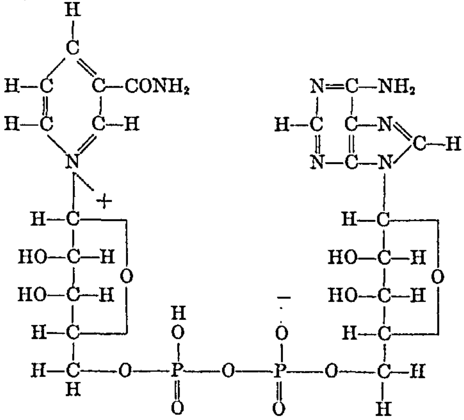
The separation of the nucleotides depends on the unequal solubilities of their barium salts. The barium salt of adenine nucleotide is insoluble in water, the barium salt of triphosphopyridine nucleotide is insoluble in dilute alcohol, whereas the barium salt of diphosphopyridine nucleotide is soluble in dilute alcohol. After separation, each fraction is then subjected to further purification. DPN is precipitated with mercury acetate, followed by precipitation with acetone, and finally by precipitation with ethyl acetate out of acid methanol solution.

Chemistry of the pyridine nucleotides. The pyridine nucleotides (DPN, TPN) are soluble in water, stable at 80°C., and readily diffuse through membranes (3, 59, 60). Their molecular weight is about 700. They are insoluble in the fat solvents, and can be precipitated by them, for example, by the addition of ethyl acetate to an aqueous methanol

solution (16). These coenzymes readily form salts with Ba, Pb, Hg, Ag, and Zn, (16, 21, 53–56, 59, 61–63) many of the salts being insoluble in water or in dilute alcohol. The coenzymes can also be precipitated with phosphotungstic acid (59) or tannic acid (59, 64). Aqueous solutions of the pyridine nucleotides are acid in reaction (16) and in general the oxidized forms are much more stable in acid than in alkali (16, 20, 65–68). The reverse is true of the reduced forms (16, 19, 50, 57, 65, 69, 70). The coenzymes are rapidly destroyed by ultraviolet light (71).

The coenzymes can be absorbed on Al_2O_3(61–63), triphosphopyridine nucleotide being held more firmly than diphosphopyridine nucleotide (58). This makes it possible to separate the 2 coenzymes by chromatographic means, since they can be eluted with H_3PO_4 (72). The 2 nucleotides can also be separated by virtue of the unequal solubility of their barium salts in dilute alcohol (16).

The structure of the pyridine nucleotides has been established by Warburg (16, 20) and Euler (72) and their co-workers. Diphosphopyridine nucleotide yields the following products on hydrolysis: 1 mol. of nicotinic acid amide, 1 mol. of adenine, 2 mols. of pentose, and 2 mols. of phosphoric acid. The pentose appears to be ribose (16, 20, 73–75), though it is possible that only one of the sugars is a pentose and the other a hexose (20). Euler and Schlenk visualize the structure as follows: (72)



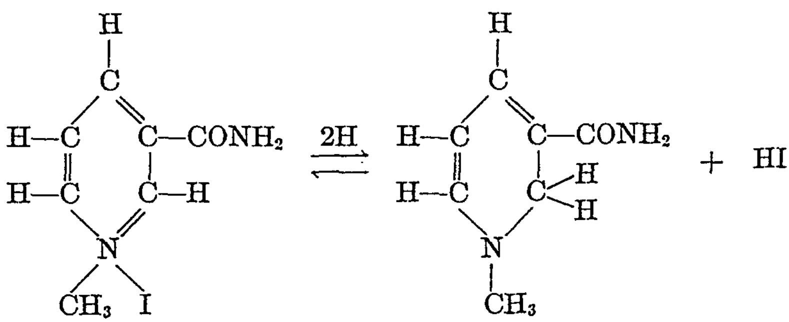
Diphosphopyridine nucleotide—coenzyme I

The empirical formula indicates that the components are connected by the removal of 5 molecules of water. Triphosphopyridine nucleotide has a similar structure except that 3 phosphoric acid residues are present in the molecule instead of 2 (16). The 7 components are joined by the removal of 6 molecules of water. The diphosphopyridine nucleotide has been converted into triphosphopyridine nucleotide by treatment with $POCl_3$(76), or enzymatically (77–80).

The most reactive part of the molecule, both chemically and biologically, is the nicotinic acid amide residue, which readily and reversibly takes on hydrogen (16, 20). Both nucleotides are easily reduced by sodium hydrosulfite, forming the dihydrids of the coenzyme.

In alkaline solution a transient yellow color appears on reduction (65) suggestive of an intermediate stage corresponding to the semiquinones of Michaelis (81). Karrer and Benz (82) observed the transient yellow color with certain "model" compounds, and found the reaction to be characteristic for pyridines able to undergo reversible reduction. The dihydrides give off a strong white fluorescence when exposed to ultraviolet light; furthermore, they have a strong absorption band in the ultraviolet with a maximum at 340 mμ. In the oxidized form the coenzymes have neither property, which therefore can be used to identify the reduced form.

Similar spectroscopic properties have been observed in certain pyridine derivatives such as trigonellin, the methyl betaine of nicotinic acid (16, 20) or the iodo-methylate of nicotinic acid (20, 83, 84). In the presence of sodium hydrosulfite the following reaction takes place.



Nicotinic acid amide iodo methylate

Reduced form

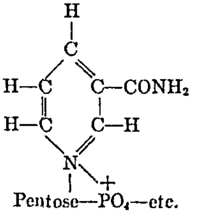
The double bond between the nitrogen of the ring and the α-carbon atom becomes saturated with the liberation of HI, the N changing from

a valence of 5 to one of 3. The reduced compound fluoresces, and has an absorption spectrum similar to that of the reduced enzymes D.P.N. and T.P.N. after treatment with sodium hydrosulfite, or reduction in an enzyme system in the presence of suitable substrates and dehydrogenases. A similar reaction is therefore postulated in all 3 cases. The reduced coenzymes D.P.N. or T.P.N. as well as the model pyridine derivatives are readily re-oxidized by shaking with methylene blue, or with the yellow enzyme in the presence of air. In fact the reduced pyridines are extremely potent reducing agents, $AgNO_3$, K_3FeCN_6, and indigo-di-tri- or tetra-sulfonate being instantly reduced in the cold (85). They can even be determined quantitatively by titration with ferricyanide (84). The reduced coenzymes are stable in alkali and unstable in acid, the reverse of the oxidized form. When the coenzymes are reduced with molecular hydrogen in the presence of Pt, 6 instead of 2 atoms of hydrogen are absorbed, and the reduction is irreversible.

The mode of action of the pyridine coenzymes. The function of the pyridine nucleotides in enzyme reactions was originally elucidated for triphosphopyridine nucleotide, in a reaction by which hexose monophosphate (Robison ester) was oxidized by molecular oxygen to phosphohexonic acid. The system consisted of a specific dehydrogenase (Zwischenferment) the coenzyme TPN, and the "yellow enzyme" (flavo protein) in addition to the substrate and oxygen. The mechanism of the reaction was revealed by studying various combinations of the components of the system (15, 16, 20).

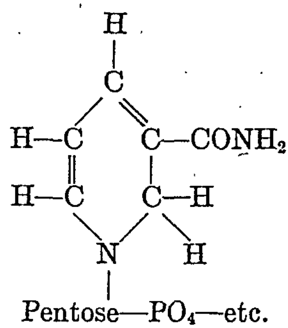
The first reaction took place when the substrate and the coenzyme were mixed in the presence of the dehydrogenase. The substrate lost two atoms of hydrogen, and the coenzyme gained two, the products of the reaction being phosphohexonic acid and reduced coenzyme.

$C_6H_{11}O_6 \cdot H_2PO_3$ +



+ H_2O →

Hexosemonophosphate + coenzyme

$C_6H_{11}O_7 \cdot H_2PO_3$ +  + H^+

phosphohexonic acid + reduced coenzyme

The reaction proceeded quantitatively and in stochiometric proportions, and stopped when no more coenzyme was present to act as hydrogen acceptor.

The hydrogen was "accepted" by the pyridine ring of the coenzyme molecule in a manner exactly analogous to that already described for the "model" pyridine compounds. The double bond between the α-carbon atom and the nitrogen of the ring became saturated, one hydrogen atom adding to the carbon of the ring, the other going to form acid with the anion released as the valence of the nitrogen changed from 5 to 3. The reaction took place either in the presence or absence of air provided the three components only—substrate, coenzyme, dehydrogenase—were present. The physical phenomena characteristic of reduced coenzymes appeared, absorption band with a maximum at 340 mμ, white fluorescence under ultra violet light.

The second reaction was studied in the absence of air. To the system—substrate, coenzyme, dehydrogenase, or its reaction products—yellow enzyme (flavoprotein) was added. A reaction took place between the reduced coenzyme and the flavoprotein:

reduced coenzyme + flavoprotein
→ regenerated coenzyme + reduced flavoprotein

The hydrogen originally coming from the substrate was thus transferred to the yellow enzyme, specifically to the alloxazine ring or the flavin part of the yellow enzyme. The coenzyme returned to its original state, becoming available to react with more substrate. Since the reactions proceeded very rapidly, only a small amount of coenzyme was necessary in the system. Hydrogen was transferred from the substrate through the coenzyme, to the flavoprotein. The over-all reaction was quantitative and stochiometric, and continued until all the sub-

strate was oxidized to phosphohexonic acid, or until all the flavoprotein was reduced to the leuco form.

If air was added to the system, a reaction took place between the leuco, or reduced, flavoprotein, and molecular oxygen:

$$\begin{array}{lclcl} \text{flavoprotein}\cdot 2\text{H} & + & O_2 & \longrightarrow & \text{flavoprotein} & + & H_2O_2 \\ \text{Reduced} & + & \text{oxygen} & \longrightarrow & \text{regenerated} & + & \text{hydrogen} \\ \text{flavoprotein} & & & & \text{yellow enzyme} & & \text{peroxide} \end{array}$$

Thus the hydrogen originally coming from the substrate was united to molecular oxygen. The yellow enzyme was regenerated in a manner exactly analogous to the regeneration of the coenzyme. Hence in the presence of molecular O_2, only a small amount of yellow enzyme needed to be present, the over-all reaction being between the substrate and molecular oxygen. Summarizing,

$$\text{Substrate} + H_2O + \text{Coenzyme} \rightarrow \text{Coenzyme}\cdot 2\text{H} + \text{oxidized substrate}$$

$$\text{Coenzyme}\cdot 2\text{H} + \text{Flavoprotein} \rightarrow \text{Coenzyme} + \text{Flavoprotein}\cdot 2\text{H}$$

$$\text{Flavoprotein}\cdot 2\text{H} + O_2 \rightarrow \text{Flavoprotein} + H_2O_2$$

The over-all reaction is:

$$\text{Substrate} + H_2O + O_2 \rightarrow \text{Oxidized substrate} + H_2O_2$$

Both coenzyme and flavoprotein were reduced and regenerated many times, and were not used up in the reaction.

The above analysis indicates the mechanism of coenzyme action, and the general phenomenon of hydrogen transport. The reactions which take place within the living cell, however, represent a more complex extension of these basic principles. In place of flavoprotein, many compounds function in transporting hydrogen from reduced coenzyme to molecular oxygen, notably the coenzyme factor (86), diaphorase (87), cytochromes (88), and cytochrome oxidase (89). Various other possible "intermediates" have been postulated. Many substrates are broken down in the living cell, each requiring a specific dehydrogenase. In addition, there are at least two pyridine coenzymes present: diphosphopyridine nucleotide, and triphosphopyridine nucleotide.

The oxidation product of any one enzymic reaction may serve as a substrate in further oxidations. Thus the phosphohexonic acid formed in the oxidation of hexosemonophosphate by triphosphopyridine nucleotide plus the specific protein component (dehydrogenase or

zwischensubstance), is readily fermented by yeast (16). Lipmann (90) studied this breakdown of phosphohexonic acid by yeast maceration juice and found that CO_2 formation took place only in the presence of oxygen. He suggested that phosphohexonic acid might represent the primary product of carbohydrate oxidation by a path differing from the usual fermentation breakdown. Warburg and Christian (91, 92) also found that phosphohexonic acid in the presence of TPN, yeast protein, and yellow enzyme could be oxidized further. Three mols of O_2 were absorbed and three mols of CO_2 were produced per mol of hexose, hence one-half of the hexosemonophosphate was oxidized to H_2O and CO_2.

Dickens (93, 94) has studied in some detail the further oxidation of phosphohexonic acid, and finds that triphosphopyridine nucleotide takes an important part. Thus it appears that TPN not only can bring about the oxidation of hexosemonophosphate to phosphohexonic acid, but in the presence of suitable protein may carry the oxidation further.

Both diphosphopyridine nucleotide and triphosphopyridine nucleotide undergo the same type of reaction (20). Each is capable of reacting with several substrates and their specific dehydrogenases. Most enzymic systems are specific in their requirement of coenzyme; that is, they require either DPN or TPN, and one coenzyme cannot replace the other. An apparent exception is the glucose dehydrogenase system of liver (95–98) which can use either DPN or TPN. Citricodehydrase also may be able to use either coenzyme (97, 99).

The best known system requiring triphosphopyridine nucleotide exclusively is the dehydrogenase of hexose monophosphate. Many systems function only in the presence of DPN; viz., those for the dehydrogenation of lactate (11, 12, 66, 99–105), malate (26, 102, 106), glutamate (107, 108), and β-hydroxy butyrate (109). Probably the most characteristic reactions of DPN are those concerned with yeast fermentation. Cozymase (DPN) has been found to function in the enzymic dehydration of hexose diphosphate (110, 111), in the fermentation of hexose diphosphate (112, 113) and in the dehydration of triose phosphates to form phosphoglyceric acid (20, 50, 114). Furthermore cozymase is the coenzyme of alcohol dehydrogenase (57, 95, 104, 116, 117) and also functions in the mutation of aldehyde (118–121) and other compounds (114–122) in which case it may be called a "co-mutase." Certain dehydrogenase systems can be "linked" with others by means of DPN (123, 124), as for example the hydroxybutyrate-acetoacetate

and the oxaloacetate-malate systems. In the presence of the respective dehydrogenases and DPN the following reactions take place:

$$\beta \text{ hydroxybutyrate} + \text{DPN} \rightarrow \text{acetoacetate} + \text{reduced DPN}$$

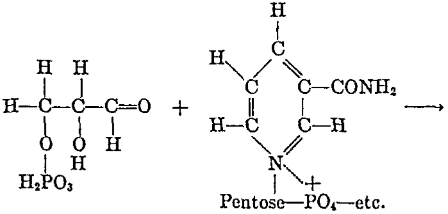
$$\text{reduced DPN} + \text{oxaloacetate} \rightarrow \text{DPN} + \text{malate}$$

the over-all reaction being:

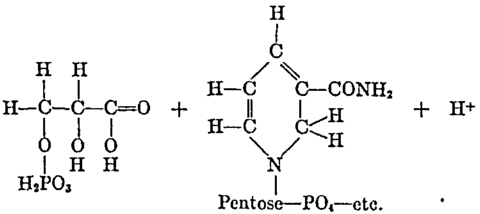
$$\beta \text{ hydroxybutyrate} + \text{oxaloacetate} \rightarrow \text{acetoacetate} + \text{malate}$$

The DPN undergoes a cycle of reduction by one dehydrogenase system and oxidation by the other.

The rôle of the coenzyme diphosphopyridine nucleotide in fermentation is perhaps best illustrated by considering a simplified form of the fermentation process. Hexose is phosphorylated to hexose diphosphate, cleaved to triose phosphate, which is then oxidized by the removal of 2 atoms of H (20, 125). Using glyceric aldehyde phosphate as a typical triose phosphate, the reaction is:



Glyceric aldehyde phosphate + Coenzyme



Glyceric acid phosphate + reduced coenzyme

The proper specific dehydrogenase must be present in addition to substrate and coenzyme (protein A in Warburg's Gär test). The reaction of the coenzyme in this stage of the fermentation process is no different than in any other dehydrogenation. However, in fermentation, the ultimate fate of the coenzyme hydrogen differs from that of hydrogen in an ordinary oxidizable system. The phosphate of glyceric acid presumably rearranges, loses phosphate and then CO_2, going to pyruvic acid, and then acetaldehyde, which acts as a hydrogen acceptor to the reduced coenzyme, the products of the reaction being ethyl alcohol and regenerated coenzyme.

For this reaction also a specific protein must be present (Warburg's protein B). This reaction, the hydrogenation of CH_3CHO, is the reverse of the dehydrogenation of ethyl alcohol for which DPN is also needed as the coenzyme (57, 95, 104, 116, 117).

The similarity between alcoholic fermentation and lactic acid formation (glycolysis) in animal tissues has long been recognized (126, 127). This similarity extends to the rôle of the coenzyme DPN in the two processes. Meyerhof and co-workers (125, 128–131) have shown that DPN acts as a hydrogen acceptor in the conversion of triose phosphate to phosphoglyceric acid. In the later stages of glycolysis, the reduced coenzyme acts as a hydrogen donator, converting pyruvic acid to lactic acid.

In addition to its action as a hydrogen carrier, diphosphopyridine nucleotide may also function in the transfer of phosphate, resembling adenylic acid in this respect (132–134). Since one-half of the diphosphopyridine nucleotide molecule is adenylic acid, this property is not surprising. Thus Ohlmeyer and Ochoa have shown that DPN is an intermediate in the transfer of phosphate from phosphopyruvic acid to glucose (135).

Phosphate transfer by means of coenzymes is remarkably sensitive to inorganic ions. Sodium ions inhibit the transfer of phosphate by either DPN or adenylic acid, but more so in the former (28, 135) and this inhibition can be counteracted by the addition of manganese. If the reaction is carried out in solutions of potassium or ammonium salts (in place of the usual sodium salt solutions) phosphate transfer will take place even in the absence of manganese. Magnesium has long been known necessary for phosphate transfer by means of adenylic acid.

The exact rôle of these various ions is still obscure, nor is the mechanism known by which diphosphopyridine nucleotide transfers phosphate. Apparently the coenzyme need not first be broken down to adenylic acid (135). It is possible that DPN takes on phosphate from

a phosphate donator, thereby changing to TPN which then passes the phosphate to a phosphate acceptor. The fact that diphosphopyridine nucleotide can be converted to triphosphopyridine nucleotide with enzymes (77, 70), microörganism (136), as well as with chemicals (76), might be considered presumptive evidence in favor of the above view, but the apparent specificity of DPN and TPN in characteristic systems suggests that these two factors are not readily interconvertible. Further study of this phase of coenzyme action will no doubt yield interesting results.

The pyridine coenzymes are essential for the growth of certain microorganisms. With Haemophilus parainfluenzae, Lwoff and Lwoff (136) have shown that "growth factor V" may be replaced by pure DPN or TPN. Adenylic acid, nicotinic acid, nicotinic acid amide, and o-dihydropropyl nicotinicamide are unable to serve as "factor V." Using the growth of H. influenzae, they have devised an extremely sensitive test for the phosphopyridine nucleotides of which they can detect 0.004 microgram. Using cultures of the bacteria grown under conditions in which "V factor" is the only limiting factor, they found that glucose, hexosemonophosphate, pyruvate, fumarate, malate, ethyl alcohol, asparagine and valine were all oxidized very slowly. Addition of either of the pyridine coenzymes brought about a rapid oxidation with all of the substrates. They found that the reaction pyridine nucleotide diphosphate ⇆ pyridine nucleotide triphosphate is possible within the bacteria.

The Lwoff test for the pyridine coenzymes has been applied to the blood of normal persons and pellagrins (137, 138). Kohn reports an increase in cozymase in both normal and pellagrous blood on administration of nicotinic acid (137). Spies found a decreased content of cozymase in the blood of both pellagrins and diabetics.[2] This can be corrected by giving nicotinic acid in either case, or in the diabetic, insulin also. This recalls an early controversy over the alleged identity of insulin and cozymase (139–144).

Cocarboxylase—thiamin pyrophosphate. *Nomenclature and history.* The existence of this coenzyme was first described by Auhagen (22) working in Euler's laboratory. He found that when dried bottom yeast was washed with phosphate buffer of pH 7.8 the entire coenzyme system was removed. The consequent loss of fermentation ability of the yeast could be restored by the addition of boiled yeast extract, but not by addition of a mixture of purified D.P.N. (cozymase), Mg, hexose diphosphate, and inorganic phosphate. This suggested that boiled yeast juice contained a second coenzyme. It was found that this

[2] Personal communication.

second coenzyme was a part of the carboxylase enzyme system and hence was termed cocarboxylase. Cocarboxylase had escaped previous detection because in fermentation studies, the yeast had not received the alkaline wash necessary to remove it. It was also a contaminant of impure cozymase preparations.

A relationship between cocarboxylase and vitamin B_1 was suggested by Simola (38) when he reported that the cocarboxylase content was decreased in the liver and brain of rats on a vitamin B deficient diet.

Lohmann and Schuster (145, 146) discovered the chemical composition of cocarboxylase and found that it was composed of 1 molecule of thiamin (vitamin B_1) and 2 molecules of phosphoric acid combined as the pyrophosphate. It is thus correct to refer to it as thiamin pyrophosphate, though cocarboxylase is the term in most general use.

Occurrence. Besides yeast, Auhagen (147) has reported the presence of cocarboxylase in liver, kidney, heart and blood. None was found in muscle, though recently its presence in small amounts has been reported there (148, 149). Its presence in rat brain and liver was observed by Simola (38). Tauber (150) reports the presence of cocarboxylase in a large number of vegetables. In animal tissues the largest amounts are found in liver and kidney (149). As carboxylase is widely distributed in nature, it is probable that cocarboxylase is similarly distributed. To what extent the thiamin in animal and vegetable tissues exists in the form of cocarboxylase is a disputed question. Undoubtedly the amount varies with species and with time though an increasing amount of experimental evidence suggests that cocarboxylase is predominant.

Preparation. Auhagen's first preparation (22) was that of a concentrate prepared by addition of lead acetate to dialyzed boiled yeast juice. The precipitated lead salts were decomposed by H_2S, and the filtrate fractionated by treatment with barium. A modification and extension of this preparation from boiled yeast juice was given by Auhagen (147) with which he prepared a concentrate 200 times more active than the yeast juice. The cocarboxylase was not prepared in pure form.

Lohmann and Schuster (146) prepared pure crystalline cocarboxylase from brewer's yeast. The cocarboxylase was extracted from the yeast by boiling water and precipitated from alkaline solution by barium. A mixture of dilute HNO_3 and H_2SO_4 was used to elute the coenzyme from the barium precipitate. Further purification was accomplished by precipitation with ethanol and methanol, adsorption on Frankonite KL and elution with pyridine, fractional precipitation with methanol ether, precipitation with picric acid, and then precipitation with phos-

photungstic acid and crystallization of the hydrochloride. One hundred kilograms of yeast yielded from 700 to 800 mgm. of the crystalline hydrochloride of cocarboxylase.

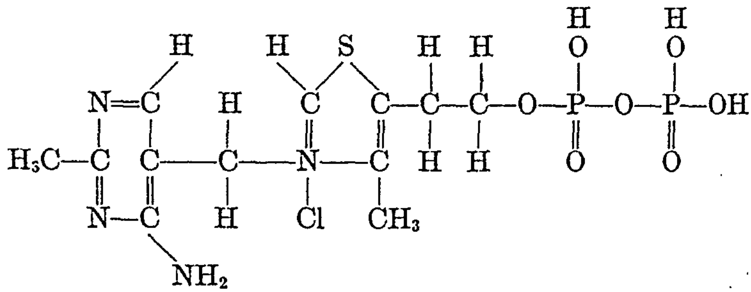
Cocarboxylase has been synthesized from thiamin by enzymatic and chemical methods. Kinnersley and Peters (151) report its synthesis by live yeast. Euler and Vestin (152) observed synthesis of cocarboxylase by the interaction of inorganic phosphate, adenosine triphosphate, and aneurin (vitamin B_1) in the presence of dried bottom yeast. Tauber (153) reported its synthesis from thiamin and orthophosphate by means of an enzyme of the duodenal mucosa of the pig. Ochoa and Peters (148) were unable to synthesize cocarboxylase with intestinal mucosa, but succeeded with liver. Stern and Hofer (154, 155) synthesized cocarboxylase by phosphorylation of vitamin B_1 with phosphorous oxychloride. Tauber (156) and Weijlard and Tauber (157) report its chemical synthesis by the interaction of thiamin hydrochloride and a mixture of $Na_2P_2O_7$ and H_3PO_4. They give a series of experiments to show that synthetic cocarboxylase as obtained from synthetic thiamin chloride is in every respect identical with cocarboxylase prepared from yeast.

Determination. The usual method of determination is that of Lohmann and Schuster (145). One gram of dried yeast is well washed with warm M/10 Na_2HPO_4 solution, and with distilled water. The residue is suspended in 10 cc. of M/10 phosphate, pH 6.2. One cubic centimeter of this washed yeast suspension and 0.4 cc. of the cocarboxylase solution are placed in the main chamber of a manometric vessel. Three tenths cubic centimeter of a sodium pyruvate solution, pH 6.2, containing 5.0 mgm. of pyruvic acid, and 0.1 mgm. of Mg, as $MgCl_2$ are placed in the side arm. After temperature equilibrium, the contents of the side arm are poured into the vessel and the cubic millimeter of CO_2 produced is observed. Sample figures taken from Lohmann and Schuster (145) are: without addition of cocarboxylase 25 cmm. of CO_2 are produced in 30 minutes, after addition of 1.0 microgram of cocarboxylase 151 cmm. of CO_2 are produced.

Westenbrink and Goudsmit (158, 159) have applied the thiochrome reaction to the determination of cocarboxylase and thiamin separately in animal tissues. The principle of the method (149) is that the free thiochrome formed by oxidation of thiamin by potassium ferricyanide in alkaline aqueous solution is extracted by isobutanol, whereas the thiochrome pyrophosphate formed from the cocarboxylase remains in the aqueous alkaline solution. Both solutions are irradiated with ultraviolet light and the intensity of fluorescence measured, from which

the concentration of thiamin and cocarboxylase are calculated. Improvements in this method have recently been made (160).

Chemistry and mode of action. Cocarboxylase is thiamin pyrophosphate; structurally it is represented as follows:



Thiamin pyrophosphate—cocarboxylase

As with inorganic pyrophosphate, one molecule of phosphate is readily removed by acid hydrolysis. The other is more resistant. Alkaline oxidation with ferricyanide produces a phosphorus-containing product with blue fluorescence in ultra violet rays (thiochrome reaction). The absorption spectrum shows 2 bands with a maximum at 245, and 260 mμ which is the same as given for aneurin (vitamin B_1 or thiamin). Cocarboxylase is an amphoteric substance. Cataphoretic experiments with the Theorell apparatus show a migration toward the cathode at a pH more acid than 3.8, and toward the anode at a pH of less acidity.

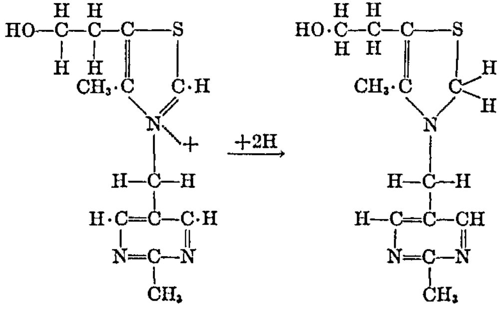
The decarboxylation of pyruvic acid in yeast by carboxylase and cocarboxylase is according to the following equation:

$$CH_3\,CO\,COOH \rightarrow CH_3\,CHO + CO_2$$

According to Neuberg and Hirsch (161) a small amount of acetoïn is produced. These products have been isolated by Lohmann and Schuster (146) from the system devised for determining cocarboxylase. They also found that acetaldehyde inhibited the enzymatic activity whereas magnesium and manganese increased the activity. Zinc and copper, on the contrary, inhibited cocarboxylase activity at higher concentrations. The optimum pH for this enzyme system is 6.3.

Lohmann's discovery that cocarboxylase contained thiamin was of great interest because it offered a definite function for vitamin B_1. A decrease in the cocarboxylase content of the tissues had previously been observed in B_1-deficient animals (38). Lohmann and Schuster (146) showed that cocarboxylase could replace vitamin B_1 in the treatment of B_1-deficient pigeons, and that it was active in the catatorulin test of Peters. Pyruvic acid accumulates in the blood and brain in

vitamin B_1 deficiency (162, 164). Disappearance of pyruvic acid and normal carbohydrate metabolism is restored upon addition of the vitamin (163–165). As cocarboxylase contains this vitamin, and definitely functions in the decarboxylation of pyruvic acid in yeast, the hypothesis was advanced that in animal tissues vitamin B_1 serves as a precursor for cocarboxylase and that it likewise functions in decarboxylating pyruvic acid. However, it has been shown (166–168) that animal tissues and certain bacteria do not decarboxylate pyruvic acid in the direct manner that yeast does, but instead oxidize it to acetic acid and CO_2, either by dismutation under anaerobic conditions (with 1 molecule being reduced to lactic acid) or directly. Lipmann (169) finds that acetone preparations of B. delbrückii can dehydrogenate pyruvic acid to acetic acid and CO_2, and yet not carry out direct decarboxylation, and regards cocarboxylase as the prosthetic group of the pyruvic acid dehydrogenating enzyme. The presence of free phosphate is necessary for the dehydrogenation; arsenate will work equally well. The dehydrogenation is independent of any dismutation. Lohmann and Schuster (146) had suggested that cocarboxylase as a prosthetic group may combine with one protein in yeast and with others in other tissues, and it may be that in Lipmann's observations it is combined with a protein component forming a pyruvic acid dehydrogenase. In this connection it is of interest to note Lipmann's observations (170, 171) on the hydrogenation of thiamin. He found that by hydrogenation with sodium hydrosulfite, one molecule of thiamin took up 2 atoms of hydrogen, and concluded that the hydrogenation takes place at the double bond adjoining the quaternary nitrogen in the thiazole part of the molecule:



Thiamin Reduced thiamin

During the reduction he observed a yellowish-green intermediate, suggestive of 2 step reduction (81). The reduction of thiamin by hydrosulfite was not reversible, inasmuch as sulfite from the $Na_2S_2O_4$ cleaves thiamin (172). In tissues, however, a reversible dehydrogenation of the thiazole ring is conceivable if the hydrogenated thiamin is stabilized (171), possibly by combination with a specific protein (dehydrogenase).

Of importance is the relationship between thiamin and cocarboxylase in animal tissues. Lipschitz, Potter and Elvehjem (173, 174) have demonstrated the simultaneous existence of both compounds in tissues and suggest that the two compounds may exist in dynamic equilibrium. They have shown that the vitamin may be phosphorylated by brewer's yeast which has been washed free of cocarboxylase with alkaline phosphate buffer, provided hexosediphosphate, vitamin B_1 and certain thermostable factors present in boiled tissue extracts are present. This synthesis was blocked by iodoacetate and it was suggested that phosphorylation occurred as a result of the esterification of inorganic phosphate coupled with the energy derived from the dismutation between triosphosphate and pyruvic acid or acetaldehyde. Ochoa and Peters (148) have objected to this work and have claimed that synthesis was not achieved by these methods. They have found that with enzyme preparations from baker's yeast there is considerable augmentation of carbon dioxide production from pyruvic acid as a substrate when vitamin B_1 is added to cocarboxylase, even in the presence of iodoacetic acid. They conclude that the increased carbon dioxide production noted in the experiments of Lipschitz et al. is due simply to activation of cocarboxylase present in the boiled tissue extract by the vitamin B_1 which is added. Apparently the discrepancy is due to the fact that "activation" of cocarboxylase is dependent upon the type of yeast used as a cocarboxylase free enzyme source.[3]

Summarizing, cocarboxylase functions in yeast with the enzyme carboxylase in decarboxylating pyruvic acid. In B. delbrückii it is a part of the system which dehydrogenates pyruvic acid. In animal tissues its precise mode of action is unknown, though it is intimately connected with thiamin and the metabolism of pyruvic acid.

Coenzyme of the D-amino acid oxidase. *Nomenclature and history.* An enzyme capable of oxidizing the enantiomorphs of many of the naturally occurring amino acids was discovered by Krebs (25, 175). The enzyme brought about the reaction:

$$R \cdot CH(NH_2) \cdot COOH + O \rightarrow R \cdot CO \cdot COOH + NH_3$$

[3] Lipton and Elvehjem, personal communication.

and was named d-amino acid oxidase or deaminase. Das (23, 24), working in Euler's laboratory, showed that the amino acid (proline) oxidase of Krebs requires a thermostable factor for its activity. Warburg and Christian (176) found that the d-amino acid oxidase of Krebs is a conjugated protein and consists of a protein and a coenzyme moiety, the latter being diffusible through cellophane. Together, the protein and coenzyme catalyze the oxidation of d-alanine to pyruvic acid by molecular oxygen; each alone is inactive. Straub (177), working in Keilin's laboratory, had likewise been studying this coenzyme, and found that the flavin content of his coenzyme preparations was proportional to their activity, suggesting that flavin is a part of the coenzyme. Simultaneously Warburg and Christian (178) found that flavin was a part of the coenzyme molecule. In a series of short papers (179–181) from Warburg's laboratory, culminating in a complete review of their work on this coenzyme (182) it has been shown that the coenzyme of the d-amino acid oxidase is alloxazin-adenin-dinucleotide. Other names that have been used (all by Warburg) for this coenzyme are: coferment of the d-alanine dehydrase, coferment of d-alanine oxidase, and the prosthetic group of the d-amino acid oxidase.

Occurrence. The coenzyme of the d-amino acid oxidase has been found in many animal tissues and in yeast. Straub used horse heart for the preparation. Warburg reports its presence in all tissues so far examined, namely: heart, liver, kidney, and tumor (Jensen-Sarcoma) of the rat, in addition to heart and muscle of the horse, and bakers' yeast. It may well be a ubiquitous substance. Warburg points out (182) that this coenzyme is probably associated with other enzymes, because there appears to be no parallelism between the content of the coenzyme and the d-amino acid oxidase in various tissues. For example, horse liver contains much coenzyme but no amino acid oxidase.

From 2½ kgm. of heart muscle, Straub (177) obtained about 12 mgm. of a yellow powder very active as coenzyme. He did not report the isolation of the pure coenzyme. The muscle was minced, washed and extracted with an acetone-water mixture. After removal of an acetic acid precipitate, the coenzyme was precipitated by further addition of acetone. The precipitate was dissolved in water and reprecipitated with barium. The barium was removed and the coenzymes precipitated by adding 10–15 volumes of acetone. The yellow powder thus obtained was soluble in water, but insoluble in organic solvents.

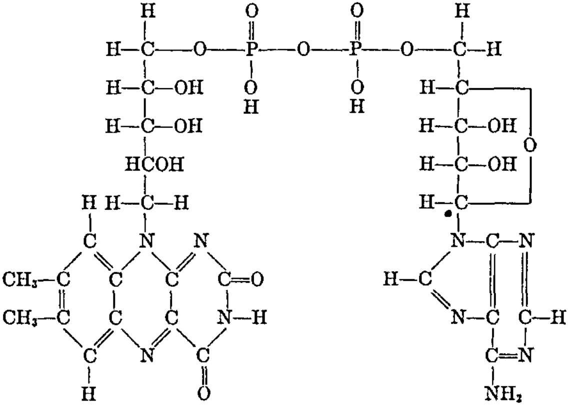
Detailed procedures for the preparation of the pure coenzyme, alloxazine-adenine-dinucleotide, from liver, kidney, and from yeast are

given by Warburg (180, 182). After extraction of the animal tissues with acetone and alcohol, the dinucleotide is extracted with water and precipitated with mercury sulfate. After removal of the mercury the dinucleotide is fractionally precipitated by silver. The silver precipitate is decomposed and the dinucleotide precipitated with barium. After removal of the barium the dinucleotide is extracted into paracresol and passed back into an aqueous solution. The pure orange colored barium salt of the dinucleotide is now carefully precipitated. From 50 kgm. of kidney or liver approximately 65 mgm. of the pure substance were obtained. A much better yield was obtained from yeast. The dinucleotide from yeast was identical with the dinucleotide prepared from kidney or liver.

Properties (182). Neutral aqueous solutions of this coenzyme retain their activity for considerable time if kept cold. The dinucleotide may be precipitated by addition of organic solvents. It may be extracted from aqueous salt solutions (NH_4SO_4 solution) by p. cresol. Normal HCl at room temperature slowly destroys the activity; at a temperature of 100° HCl destroys the activity completely in 5 minutes. N/10 H_2SO_4 or HNO_3 have little effect at room temperature. A half normal NaOH solution will completely destroy the activity in 2 hours at room temperature. When in alkaline solution, exposure to light readily inactivates the coenzyme (lumiflavin reaction); in neutral aqueous solution, exposure to light is without effect. Reversible reduction with loss of the yellow color may be accomplished with $Na_2S_2O_4$ at pH of 7.5. The dinucleotide forms insoluble salts with Hg, Ag, Pb, and Ba. The dinucleotide may be absorbed on Al_2O_3 and eluted with bicarbonate solution at pH 9.

Method of determination. The usual method of determination of this coenzyme is to determine its effectiveness in oxidizing d-alanine. An oxidation system is constructed in which the coenzyme is the limiting factor and in which its effectiveness may be observed by measuring the O_2 uptake, or by measuring the decolorization of methylene blue. For example (182) 2 cc. of a phosphate containing enzyme solution, pH 8.3 was placed in a respiration flask; d-alanine was placed in the side arm. An additional 1.34 microgram of the coenzyme was added to a second flask. Addition of the d-alanine to the first flask caused no oxygen uptake and in the second flask 22.9 cmm. of O_2 was taken up in 10 minutes. With a similar set up, Straub (177) found that in the presence of the coenzyme, methylene blue was reduced in 27 minutes, and without coenzyme it was not reduced even in 12 hours.

Chemistry and mode of action. The most significant event in the chemistry of this coenzyme was the isolation by Warburg (178) of an orange colored barium salt, which gave the absorption spectrum characteristic of flavin, and which on alkaline photolysis yielded one molecule of lumiflavin per atom of barium, and on acid hydrolysis yielded one molecule of adenine per atom of barium. Further work from Warburg's laboratory showed that the coenzyme was alloxazine-adenine-dinucleotide. Elementary analysis of the pure barium salt gave a molecular formula of $C_{27}H_{31}N_9P_2BaO_{15}$ (molecular wt.—920). Though Warburg assigned no structural formula to the coenzyme, one can visualize it as follows:



Alloxazine—adenine—dinucleotide

The absorption spectrum of this coenzyme is very similar to that of lactoflavin. Absorption bands appear with maxima at 200, 375, and 450 mμ. In the ultraviolet the dinucleotide gives a stronger absorption band. The dinucleotide absorbs slightly more than lactoflavin in the green region of the spectrum and hence its solutions appear reddish yellow as contrasted with the greenish yellow of lactoflavin solutions.

The mechanism of the action of the coenzyme of the d-amino acid oxidase is analogous to the action of the "old" yellow enzyme in Warburg's experiment with hexosemonophosphate and triphosphopyridine

nucleotide. The alloxazine ring of the coenzyme accepts hydrogen from the amino acid, and reduced coenzyme is formed. This then may be re-oxidized by molecular oxygen. Warburg has calculated the oxidative effectiveness of this coenzyme from the alanine test and finds that one molecule of dinucleotide may react with 1440 mols of oxygen per minute, which is theoretically equivalent to the oxidation of 2880 molecules of alanine to pyruvic acid.

ADENYLIC ACID. Another type of coenzyme, adenylpyrophosphate, plays an important rôle in muscle metabolism, since it functions both in anaerobic glycolysis and in aerobic respiration. The adenylpyrophosphate-adenylic acid system[4] has been extensively studied by Meyerhof, Embden, Parnas, Needham and others. Adenylpyrophosphate added to a dialyzed muscle extract transfers phosphate groups[5] to carbohydrate, forming hexose phosphates and adenylic acid. The hexose phosphates break down further, without the coöperation of this coenzyme system, until the phosphopyruvic acid stage. Here by another enzyme-coenzyme reaction, phosphate is transferred back to adenylic acid with the regeneration of adenylpyrophosphate. Thus, this coenzyme, also, is broken down and later regenerated, but in contrast to Warburg's coenzyme, the change involves phosphate, rather than hydrogen, and furthermore several steps are intermediate between the dephosphorylation of adenylpyrophosphate and its regeneration. (Hexose phosphate → triose phosphate → phosphoglyceric acid → phosphopyruvic acid.) Just as adenylpyrophosphate is considered a coenzyme necessary in forming the hexose phosphates from glycogen, so too, adenylic acid is a coenzyme for the enzymatic breakdown of phosphopyruvic acid to pyruvic acid. Two mechanisms exist for the regeneration of the adenylic acid, the transfer of phosphate from the adenylpyrophosphate to glycogen and the transfer of phosphate to creatin. Magnesium ions are essential for the action of the adenylpyrophosphate system. A detailed summary of the adenylpyrophosphate-adenylic acid system may be had from the reviews by Lohmann (185), Meyerhof (186) and Parnas and Ostern (187).

MISCELLANEOUS COENZYMES. Two other factors have been called

[4] The rôle of coenzymes in muscle metabolism has recently been reviewed by Dr. Dorothy Needham in Biokhimiya (183), but since this journal is not readily available, the essential facts regarding coenzymes in muscle metabolism are given here.

[5] Euler et al. (184) have suggested the term cophosphorylase for coenzymes of the adenylic acid type which function in the transfer of phosphate.

coenzymes, co-ferment T and coenzyme R. "Co-ferment T" which increases anaerobic glycolysis, is obtained from normal and malignant tissues (188). Its identity with pyruvic acid (189) has been questioned (190). "Coenzyme R", a factor needed by certain bacteria, was first extracted from commercial cane sugar (191), but is also present in many substances of plant and animal origin (192). It is dialyzable, heat stable, and is thought to play a part in the respiration of legume root nodule bacteria. Since there is no evidence that "coenzyme R" does actually function in respiration, it might better be classed as a growth factor until more data are accumulated.

Coenzyme activity has also been attributed to glutathione, Lohmann reporting that glutathione is the coenzyme for methyl glyoxalase (193). Apparently glutathione reacts with methyl glyoxal, and the compound formed serves as a substrate for methyl glyoxalase, the end product being lactic acid and regenerated glutathione. Methyl glyoxal, however, is no longer considered an intermediate in glycolysis, being regarded rather as an abnormal decomposition product of glyceric aldehydephosphate (194). Nevertheless methyl glyoxal offers a possible alternate path for lactic acid formation. Recently Morgan and Friedmann (195, 196) have observed that maleic acid forms addition compounds with thiol groups, and strongly inhibits the coenzyme activity of glutathione, and also of glyoxalase.

General discussion. The older concept of coenzymes was that of accessory substances assisting or speeding up reactions which were primarily determined by enzymes of the nondialyzable, heat labile, protein type. In line with the view that biological oxidation proceeds by dehydrogenation of the substrate, coenzymes were said to function in dehydrogenation by "labilizing" or "activating" substrate hydrogen, thus more readily permitting its removal by the dehydrogenase.

These older ideas have been modified. The isolation of natural compounds which function in oxido-reduction processes, and the study of artificial systems composed of these substances, have suggested their function in the living cell. Apparently intra-cellular oxidation is accomplished by means of a complicated chain of reactions in which hydrogen is transferred from the substrate through a series of compounds until eventually it unites with oxygen. Coenzymes function by actually uniting with hydrogen, and then passing it on to some other compound in the chain. Substances which are included in the line of hydrogen transfer are the dehydrogenases, the coenzymes, the coenzyme factor (86), the cytochromes (88), and cytochrome oxidase (89) (atmungs-

ferment or indophenol oxidase). Still other naturally occurring substances may function in some interlocking way with this chain of dehydrogenation, or perhaps offer alternate routes of transfer from substrate to oxygen, for example, yellow enzyme, certain of the dicarboxylic acids (197, 198), glutathione (199), and adrenaline (200). In fact, any naturally occurring substance that is readily and reversibly oxidized and reduced in the cell can be suspected of functioning in intracellular metabolism. Considerable variation is possible in the routes of hydrogen transfer postulated in various tissues and in the tissues of various species, but that such routes do exist appears to be generally accepted.

Coenzymes are no longer looked upon as accelerators[6] in any supplementary sense, but rather as essential, integral members of a complicated "bucket brigade," transferring hydrogen or phosphate from compound to compound; they empty their buckets and come back for more. They are "carriers" of both hydrogen and phosphate.

But protein enzymes of the traditional type, the dehydrogenases, must be present to bring about the transfer of hydrogen from substrate to coenzyme. The coöperation between enzyme and coenzyme is obviously as real today as when the word "coenzyme" was first coined, but the demonstration of hydrogen transfer, however significant, does not clarify the relationship between enzyme and coenzyme. Two points of view have been expressed. The first is that the dehydrogenase and the coenzyme, though related in function, are separate entities, and uncombined. The second is that the coenzyme unites with the protein (dehydrogenase) to form a conjugated protein, or "proteid," of which the coenzyme is the prosthetic group.

As evidence for the former view the great specificity of the dehydrogenases has been stressed. Some are specific to only one compound, others, to a class of compounds. Thus, lactic dehydrogenase acts only with lactic acid, glucose dehydrogenase with glucose, xanthine dehydrogenase with xanthine and simple aldehydes, α-glycerophosphate dehydrogenase with α-glycerophosphate, etc. The oxidation of any of these substrates takes place only in the presence of the proper dehydrogenase and a suitable hydrogen acceptor.

The action of certain specific dehydrogenases can be inhibited by

[6] Loevenhart (6) was the first to attempt a limitation of the term coenzyme. He suggested that the term "be reserved for those substances which are absolutely necessary in order that the enzyme manifest itself at all," and that substances which merely accelerate enzyme processes may be termed accelerators.

substances closely related to the substrate, as for example, malonic acid, which inhibits succinic acid dehydrogenase (20). This inhibition is explained on the basis of a combination of the dehydrogenase and the inhibitor—"tying it up." More direct evidence for the union of dehydrogenase and substrate has been obtained by cataphoresis experiments. From an analysis of the rates of migration of mixtures of hexose monophosphate, triphosphopyridine nucleotide, and the dehydrogenase in an electric field Theorell (202) concluded that the substrate formed a compound with the dehydrogenase, and also with the coenzyme. No evidence of an enzyme-coenzyme union was observed. Enzymic oxidation, therefore, might be viewed as follows:

(1) substrate + dehydrogenase → substrate-dehydrogenase complex with "activated" hydrogen

(2) substrate-dehydrogenase complex + coenzyme → oxidized substrate + dehydrogenase + reduced coenzyme.

Warburg holds a different view (203). He emphasizes the combination of protein (dehydrogenase) and coenzyme. In a sense this is a modern adaptation of the Willstaetter idea of a "colloidal carrier" and a non-colloidal "active group," but whereas the older workers attached no particular significance to the protein "carrier," Warburg, of course, appreciates its specificity. The analogy to hemoglobin has been used—the protein (dehydrogenase) corresponding to the globin, and the coenzyme to the hematin, the combination being necessary for activity. As there are many different globins in various animal species combined with the same hematin, so there can be many protein dehydrogenases combined with the same coenzyme.

Warburg's nomenclature differs from that generally used in English. The dehydrogenase of hexose monophosphate is called "zwischensubstanz," the combination of protein and coenzyme is called "enzyme."

Protein component + coenzyme → hydrogen transporting enzyme
(Zwischensubstanz)
(Dehydrogenase)

This point of view most certainly applies to the flavin enzymes. Thus Theorell (204) succeeded in splitting the yellow enzyme into a protein component and a prosthetic group by dialyzing a solution of the yellow enzyme against 0.02 N HCl for 72 hours. The prosthetic group, flavin phosphate, was dialyzed away leaving the undenatured protein component. This protein could be combined with flavin phosphate pre-

pared synthetically (205) or by methyl alcohol extraction (204), to re-synthesize an active yellow enzyme. Nonphosphorylated flavin did not form a compound with the protein of the yellow enzyme.

d-Amino oxidase, likewise, has been cleaved into a protein component and an active prosthetic group by hydrolysis with dilute HCl (182). An $(NH_4)_2SO_4$ solution of the enzyme was used. The prosthetic group was found to be the alloxazine-adenine-dinucleotide, and corresponds to the flavin-containing coenzyme previously reported (176, 177). Protein and prosthetic groups could be recombined to give the active "enzyme."

TABLE 1

	Protein component (dehydrogenase) (zwischensubstanz)	*Coenzyme* (prosthetic group) (active group)	*Enzyme* (Enzyme-complex)
1	Protein (O_2—reduced coenzyme)*	+ alloxazine nucleotide	Alloxazine-proteid† (O_2—reduced coenzyme)
2	Protein (O_2—reduced coenzyme)	+ alloxazine adenine dinucleotide	Alloxazine adenine dinucleotide proteid (O_2—reduced coenzyme)
3	Protein (methylene blue—reduced coenzyme)	+ alloxazine adenine dinucleotide	Alloxazine adenine dinucleotide proteid (methylene blue—reduced coenzyme)
4	Protein (O_2—d-amino acid)	+ alloxazine adenine dinucleotide	Alloxazine adenine dinucleotide proteid (O_2—amino acid)
5	Protein (O_2—xanthine)	+ alloxazine adenine dinucleotide	Alloxazine adenine dinucleotide proteid (O_2—xanthine)

* The subscripts in parenthesis indicate the oxidizing agent and the substances oxidized.

† Warburg uses the term "proteid" to refer to the union between the protein and prosthetic group.

A new synthetic enzyme was prepared by adding alloxazine-adenine-dinucleotide (prosthetic group or coenzyme from d-amino oxidase) to the protein component of the "old" yellow enzyme (206). The resulting compound transferred hydrogen from dihydropyridine to molecular oxygen very much as did the old yellow enzyme.

Still another flavin enzyme has been isolated by Haas (207), working in Warburg's laboratory. The enzyme was found in yeast, and also contains the alloxazine-adenine-dinucleotide as prosthetic group, but the protein component differs from that of the enzymes previously discussed. The enzyme will oxidize reduced TPN, but reacts much more rapidly with methylene blue than with molecular oxygen.

Ball, working in Warburg's laboratory, reports that xanthine oxidase, also, may be split into a protein component and a prosthetic group (208, 206). Here too the prosthetic group is alloxazine-adenine-dinucleotide, the same as the coenzyme of the amino acid oxidase and of the new yellow enzyme. As yet it has not been possible to regenerate an active xanthine oxidase.

These observations may best be summarized by the accompanying table (table 1) taken essentially from Warburg and Christian (206).

In 1 and 2 the protein component is the same and the coenzyme (prosthetic group) is different. In 2, 3, 4 and 5 the protein component differs and the coenzyme is the same. It is the protein component that gives the enzyme specificity and determines whether the coenzyme shall accept hydrogen from xanthine, from amino acids, or from the reduced phosphopyridine nucleotides.

There is, therefore, ample evidence that the alloxazine-adenine-dinucleotide combines with specific proteins to form active "enzymes." It is merely a question of nomenclature whether the protein component alone shall be called "enzyme," or whether the protein-coenzyme complex, the proteid, is called the "enzyme." Both components are capable of uniting with one another to form stable compounds. Xanthine oxidase, the classical example of an enzyme which does not require a coenzyme, is one of these stable proteids. The reason it did not appear to need a coenzyme was that the coenzyme (prosthetic group) was already present, firmly attached to the protein component. When the proper chemical procedure was found, it was possible to separate the prosthetic group. Future work may show that other enzymes, which today are considered acting without coenzymes, may be dissociated by the proper chemical treatment into a protein component and an active prosthetic group having the properties of a coenzyme.

It is quite possible that the pyridine nucleotides, also, combine with specific proteins to form an active complex. However, the resulting pyridine proteids are much more easily dissociated than the alloxazine proteids; and hence the prosthetic group (or coenzyme) can be removed more readily (209–212). Dissociation constants have been determined for the proteids of both the reduced and oxidized form of the pyridine-nucleotides, and the reduced form of the coenzyme found to be much less firmly bound than the oxidized form (211).

The enzyme-coenzyme relationship might be pictured as follows: reaction between coenzyme and substrate takes place when the proper dehydrogenase, a large molecule, seizes the coenzyme with one hand, the substrate with the other, and bumps the two together. The co-

enzyme takes hydrogen from the substrate, and the reduced coenzyme breaks away. It can then pass its hydrogen on to a suitable acceptor, either as free reduced coenzyme or in the presence of some other specific protein.

Recently Corron and Green (213) have isolated a flavo-protein from milk which markedly accelerates the oxidation of reduced DPN by methylene blue or pyocyanine. The prosthetic flavin appears to be a phosphorylated derivative, not identical with lactoflavin phosphate. It can function as the coenzyme of d-amino acid oxidase, but the authors claim that its chemical and physical properties are not identical with those of the amino acid oxidase coenzyme. They found that the milk flavo-protein does not itself become reduced during its catalysis of hydrogen transfer, and assume that it forms a complex with reduced coenzyme which accelerates the reaction with methylene blue.

The relationship between coenzymes and vitamins is particularly interesting. In both diphosphopyridine nucleotide and triphosphopyridine nucleotide the enzymatically-active group is nicotinic acid amide, which is also active as a vitamin in the cure of canine black tongue (214) and human pellagra (215). In cocarboxylase the active group is thiamin or vitamin B_1. In the coenzyme of d-amino acid oxidase the active group contains riboflavin, a member of the vitamin B_2 complex (216, 217). Thus, in addition to clarifying our knowledge of cellular metabolism, the study of coenzymes has materially enhanced our knowledge of the vitamins.

It is of interest that all of the coenzymes discussed in this review contain phosphate. Many biological reactions, particularly those involving marked energy changes, are intimately dependent upon phosphate containing compounds and frequently involve phosphate transfer. Reactions involving coenzymes are no doubt important sources of energy to the living cell. Likewise it is interesting to speculate on the fact that coenzymes are nucleotides,[7] a class of compounds characteristic of nucleoproteins and the cell nucleus. Cytologists have long maintained that the nucleus is the controlling, physiologically dominant part of the cell. Geneticists have placed the dominance of the nuclear material on the chromosomes and their genes. The demonstration of the rôle of certain coenzymes, nucleotides, in biological oxido-reductions in an interesting biochemical counterpart of the cytological interpretation of nuclear function.

[7] Though cocarboxylase as isolated in the laboratory is not a nucleotide, one can readily postulate that within the cell it exists in a "nucleotide-like" combination.

REFERENCES

(1) Bertrand, G. Compt. rend. Acad. Sci. 124: 1032, 1897.
(2) Harden, A. and W. J. Young. J. Physiol. 32: (Proc.) Nov. 12, 1904.
(3) Harden, A. and W. J. Young. Proc. Roy. Soc. B. 78: 369, 1906.
(4) v. Euler H. and K. Myrbäck. Ztschr. physiol. Chem. 131: 179, 1923.
(5) Waksman, S. A. and W. C. Davidson. Enzymes. Williams & Wilkins Company, Baltimore, 1926.
(6) Loevenhart, A. S. J. Biol. Chem. 2: 391, 1906.
(7) Onodera, N. Bioch. J. 9: 575, 1915.
(8) Sumner, J. and J. Kirk. Biochem. J. 26: 551, 1932.
(9) Meyerhof, O. Ztschr. physiol. Chem. 101: 165, 1918.
(10) Szent-Gyorgyi, A. Biochem. Ztschr. 157: 50, 1925.
(11) Banga, I., A. Szent-Gyorgyi and L. Varga. Ztschr. physiol. Chem. 210: 228, 1932.
(12) Banga, I. and A. Szent-Gyorgyi. Ztschr. physiol. Chem. 217: 39, 1933.
(13) Szent-Gyorgyi, A. Studies on biological oxidation and some of its catalysts. K. Renyi, Budapest, 1937.
(14) Warburg, O. and W. Christian. Biochem. Ztschr. 254: 438, 1932.
(15) Warburg, O., W. Christian and A. Griese. Biochem. Ztschr. 279: 143, 1935.
(16) Warburg, O., W. Christian and A. Griese. Biochem. Ztschr. 282: 157, 1935.
(17) Warburg, O. and W. Christian. Biochem. Ztschr. 238: 131, 1931.
(18) Warburg, O. and W. Christian. Biochem. Ztschr. 242: 206, 1931.
(19) Warburg, O. and W. Christian. Biochem. Ztschr. 285: 156, 1936.
(20) Warburg, O. and W. Christian. Biochem. Ztschr. 287: 291, 1936.
(21) v. Euler, H., H. Albers and F. Schlenk. Ztschr. physiol. Chem. 237: 1, 1935.
(22) Auhagen, E. Ztschr. physiol. Chem. 204: 149, 1932.
(23) Das, N. B. Bioch. J. 30: 1080, 1936.
(24) Das, N. B. Bioch. J. 30: 1617, 1936.
(25) Krebs, H. A. Ztschr. physiol. Chem. 217: 191, 1933.
(26) Green, D. E. Biochem. J. 30: 2095, 1936.
(27) Myrbäck, K. Tabul. biol. 14: 110, 1937.
(28) v. Euler, H., et al. Ztschr. physiol. Chem. 245: 217, 1937.
(29) Schlenk, F. Tabul. biol. 14: 186, 1937.
(30) Myrbäck, K. Ergeb. Enzymf. 2: 139, 1933.
(31) Myrbäck, K. and H. v. Euler. Ber. Chem. Ges. 57: 1073, 1924.
(32) v. Euler, H. and R. Nilsson. Ztschr. physiol. Chem. 162: 63, 1926.
(33) Adler, E. and H. v. Euler. Sven. Kem. Tidsk. 48: 221, 1936.
(34) v. Euler, H. and R. Nilsson. Ztschr. physiol. Chem. 155: 186, 1926.
(35) v. Euler, H. and K. Myrbäck. Ztschr. physiol. Chem. 181: 1, 1929.
(36) Sym, E., R. Nilsson and H. v. Euler. Ztschr. physiol. Chem. 190: 228, 1930.
(37) v. Euler, H. and H. Runehjelm. Ztschr. physiol. Chem. 165: 306, 1927.
(38) Simola, P. Biochem. Ztschr. 254: 229, 1932.
(39) Ochoa, S. and G. G. Ochoa. Nature, 140: 1097, 1937.
(40) v. Euler, H., F. Schlenk and N. Forsman. Naturw. 26: 11, 1938.

(41) v. EULER, H. AND S. STEFFENBURG. Ztschr. physiol. Chem. 175: 38, 1928.
(42) v. EULER, H. AND R. NILSSON. Ztschr. physiol. Chem. 194: 260, 1931.
(43) v. EULER, H. AND K. MYRBÄCK. Sven. Kem. Tidsk. 37: 173, 1925.
(44) WARBURG, O. AND W. CHRISTIAN. Biochem. Ztschr. 282: 221, 1935.
(45) WARBURG, O. AND W. CHRISTIAN. Ber. Chem. Ges. 69: 228, 1936.
(46) MYRBÄCK, K. Ztschr. physiol. Chem. 177: 158, 1928.
(47) MYRBÄCK, K. AND B. ÖRTENBLAD. Ztschr. physiol. Chem. 241: 148, 1936.
(48) v. EULER, H. AND S. KARLSSON. Ztschr. physiol. Chem. 123: 90, 1922.
(49) NEGELEIN, E. Biochem. Ztschr. 287: 329, 1936.
(50) v. EULER, H., E. ADLER AND H. HELLSTRÖM. Ztschr. physiol. Chem. 241: 239, 1936.
(51) GREEN, D. E. AND J. G. DEWAN. Biochem. J. 31: 1069, 1937.
(52) WARBURG, O., E. NEGELEIN AND E. HAAS. Biochem. Ztschr. 227: 171, 1930.
(53) v. EULER, H., H. ALBERS AND F. SCHLENK. Ztschr. physiol. Chem. 234: 1, 1935.
(54) MYRBÄCK, K. AND H. LARSSON. Ztschr. physiol. Chem. 225: 131, 1934.
(55) MYRBÄCK, K. AND B. ÖRTENBLAD. Ztschr. physiol. Chem. 233: 87, 1935.
(56) v. EULER, H., H. ALBERS AND F. SCHLENK. Ztschr. physiol. Chem. 240: 113, 1936.
(57) v. EULER, H. AND E. ADLER. Ztsch. physiol. Chem. 238: 233, 1935.
(58) OHLMEYER, P. Biochem. Ztschr. 297: 66, 1938.
(59) v. EULER, H. AND K. MYRBÄCK. Ztschr. physiol. Chem. 169: 102, 1937.
(60) MYRBÄCK, K. Ztschr. physiol. Chem. 219: 173, 1933.
(61) MYRBÄCK, K. AND H. v. EULER. Ztschr. physiol. Chem. 138: 1, 1924.
(62) v. EULER, H. AND K. MYRBÄCK. Ztschr. physiol. Chem. 136: 107, 1924.
(63) v. EULER, H. AND K. MYRBÄCK. Ztschr. physiol. Chem. 198: 219, 1931.
(64) v. EULER, H. AND K. MYRBÄCK. Ztshr. physiol. Chem. 139: 281, 1924.
(65) ADLER, E., H. HELLSTRÖM AND H. v. EULER. Ztschr. physiol. Chem. 242: 225, 1936.
(66) GREEN, D. E. AND J. BROSTEAUX. Biochem. J. 30: 1489, 1936.
(67) MYRBÄCK, K. AND R. NILSSON. Ztschr. physiol. Chem. 165: 140, 1927.
(68) WARBURG, O. AND W. CHRISTIAN. Biochem. Ztschr. 274: 112, 1934.
(69) v. EULER, H., E. ADLER AND H. HELLSTRÖM. Sven. Kem. Tidsk. 47: 290, 1935.
(70) HAAS, E. Biochem. Ztschr. 288: 123, 1936.
(71) WARBURG, O. AND W. CHRISTIAN. Biochem. Ztschr. 282: 221, 1935.
(72) v. EULER, H. AND F. SCHLENK. Ztschr. physiol. Chem. 246: 64, 1936.
(73) MYRBÄCK, K. Ztschr. physiol. Chem. 217: 249, 1933.
(74) MYRBÄCK, K. AND H. v. EULER. Ztschr. physiol. Chem. 203: 143, 1931.
(75) v. EULER, H., P. KARRER AND B. BECKER. Helv. chim. Acta. 19: 1060, 1936.
(76) SCHLENK, F. Naturwiss. 25: 668, 1937.
(77) VESTIN, R. Naturwiss. 25: 667, 1937.
(78) VESTIN, R. AND H. v. EULER. Ztschr. physiol. Chem. 247: 43, 1937.
(79) v. EULER, H. AND E. ADLER. Ztschr. physiol. Chem. 252: 41, 1938.
(80) v. EULER, H. AND S. BAUER. Ber. Chem. Ges. 71: 411, 1938.
(81) MICHAELIS, L. Chem. Rev. 16: 243, 1935.

(82) KARRER, P. AND F. BENZ. Helv. chim. Acta. 19: 1028, 1936.
(83) KARRER, P. AND O. WARBURG. Biochem. Ztschr. 285: 297, 1936.
(84) KARRER, P., G. SCHWARZENBACH, F. BENZ AND U. SOLMSSEN. Helv. chim. Acta 19: 811, 1936.
(85) KARRER, P. Helv. chim. Acta 19: E 33, 1936.
(86) DEWAN, J. AND D. E. GREEN. Biochem. J. 32: 626, 1938.
(87) ADLER, E., H. v. EULER AND H. HELLSTRÖM. Sv. Vet. Akad. Ark. f. Kemi 12: no. 38, 1937.
(88) KEILIN, D. Ergeb. Enzymf. 2: 239, 1933.
(89) KEILIN, D. AND E. F. HARTREE. Proc. Roy. Soc. B. 125: 171, 1938.
(90) LIPMANN, F. Nature 138: 588, 1936.
(91) WARBURG, O. AND W. CHRISTIAN. Biochem. Ztschr. 287: 440, 1936.
(92) WARBURG, O. AND W. CHRISTIAN. Biochem. Ztschr. 292: 287, 1937.
(93) DICKENS, F. Nature 138: 1057, 1936.
(94) DICKENS, F. Biochem. J. 32: 1626, 1938.
(95) v. EULER, H. ET AL. Ztschr. physiol. Chem. 233: 120, 1935.
(96) ADLER, E. AND H. v. EULER. Ztschr. physiol. Chem. 232: 6, 1935.
(97) ANDERSSON, B. Ztschr. physiol. Chem. 225: 57, 1934.
(98) DAS, N. B. Ztschr. physiol. Chem. 238: 269, 1936.
(99) WAGNER-JAUREGG, T., H. RAUEN AND E. F. MÖLLER. Ztschr. physiol. Chem. 228: 273, 1934.
(100) BERNHEIM, F. Biochem. J. 22: 1178, 1928.
(101) BOYLAND, E. AND M. BOYLAND. Biochem. J. 28: 1417, 1934.
(102) DAS, N. B. Biochem. J. 31: 1116, 1937.
(103) QUASTEL, J. H. AND A. H. M. WHEATLEY. Biochem. J. 32: 940, 1938.
(104) ANDERSSON, B. Ztschr. Physiol. Chem. 217: 186, 1933.
(105) WAGNER-JAUREGG, T. AND E. F. MÖLLER. Ztschr. physiol. Chem. 236: 216, 1935.
(106) OGSTON, F. J. AND D. E. GREEN. Biochem. J. 29: 1983, 1935.
(107) DEWAN, J. G. Biochem. J. 32: 1378, 1938.
(108) v. EULER, H. ET AL. Ztschr. physiol. Chem. 254: 61, 1938.
(109) GREEN, D. E., J. G. DEWAN AND L. F. LELOIR. Biochem. J. 31: 934, 1937.
(110) v. EULER, H. AND R. NILSSON. Ztschr. physiol. Chem. 149: 44, 1925.
(111) v. EULER, H. AND R. NILSSON. Biochem. J. 25: 2168, 1931.
(112) SCHAFFNER, A., E. BAUR AND H. BERL. Ztschr. physiol. Chem. 232: 213, 1935.
(113) SCHAFFNER, A., E. BAUR AND H. BERL. Ztschr. physiol. Chem. 238: 111, 1935.
(114) GREEN, D. E., D. M. NEEDHAM AND J. G. DEWAN. Biochem. J. 31: 2327, 1937.
(115) LUTWAK-MANN, C. Biochem. J. 32: 1364, 1938.
(116) ANDERSSON, B. Ztschr. physiol. Chem. 225: 57, 1934.
(117) v. EULER, H. AND E. ADLER. Ztschr. physiol. Chem. 226: 195, 1934.
(118) v. EULER, H. AND K. MYRBÄCK. Sven. Kem Tidskr. 38: 353, 1926.
(119) MYRBÄCK, K. AND W. JACOBI. Ztschr. physiol. Chem. 161: 245, 1926.
(120) v. EULER, H. AND K. MYRBÄCK. Ztschr. physiol. Chem. 165: 28, 1927.
(121) DIXON, M. AND C. LUTWAK-MANN. Bioch. J. 31: 1347, 1937.

(122) MYRBÄCK, K., H. v. EULER AND E. SANDBERG. Ztschr. physiol. Chem. 175: 316, 1928.
(123) DEWAN, J. G. AND D. E. GREEN. Bioch. J. 31: 1074, 1937.
(124) NEEDHAM, D. M. AND G. D. LU. Bioch. J. 32: 2040, 1938.
(125) MEYERHOF, O., W. KIESSLING AND W. SCHULZ. Biochem. Ztschr. 292: 25, 1937.
(126) MEYERHOF, O. Pflüger's Arch. 170: 367, 1918.
(127) MEYERHOF, O. Pflüger's Arch. 175: 20, 1919.
(128) MEYERHOF, O. AND P. OHLMEYER. Biochem. Ztschr. 290: 334, 1937.
(129) MEYERHOF, O., W. SCHULZ AND P. SCHUSTER. Biochem. Ztschr. 293: 309, 1937.
(130) MEYERHOF, O., P. OHLMEYER AND W. MÖHLE. Biochem. Ztschr. 293: 334, 1937.
(131) MEYERHOF, O., P. OHLMEYER AND W. MÖHLE. Biochem. Ztschr. 297: 113, 1938.
(132) MEYERHOF, O. AND W. KIESSLING. Naturwiss. 24: 361, 1936.
(133) MEYERHOF, O. AND W. KIESSLING. Naturwiss. 24: 557, 1936.
(134) OHLMEYER, P. Biochem. Ztschr. 287: 212, 1936.
(135) OHLMEYER, P. AND S. OCHOA. Biochem. Ztschr. 293: 338, 1938.
(136) LWOFF, A. AND M. LWOFF. Proc. Roy. Soc. B. 122: 352, 1937.
(137) KOHN, H. I. Biochem. J. 32: 2075, 1938.
(138) SPIES, T. D., R. E. STONE AND J. B. MCLESTER. South. Med. J. 31: 1231, 1938.
(139) VIRTANEN, A. I. Ber. Chem. Ges. 58: 2441, 1925.
(140) v. EULER, H., E. JORPES AND K. MYRBÄCK. Ztschr. physiol. Chem. 149: 60, 1925.
(141) v. EULER, H. AND K. MYRBÄCK. Ztschr. physiol. Chem. 150: 1, 1925.
(142) JORPES, E., H. v. EULER AND R. NILSSON. Ztschr. physiol. Chem. 155: 137, 1926.
(143) VIRTANEN, A. I. AND Q. KARSTROM. Ber. Chem. Ges. 59: 45, 1926.
(144) BRUGSCH, T. AND H. HORSTERS. Ztschr. physiol. Chem. 157: 186, 1926.
(145) LOHMANN, K. AND P. SCHUSTER. Naturwiss. 25: 26, 1937.
(146) LOHMANN, K. AND P. SCHUSTER. Biochem. Ztschr. 294: 188, 1937.
(147) AUHAGEN, E. Biochem. Ztschr. 258: 330, 1933.
(148) OCHOA, S. AND R. A. PETERS. Biochem. J. 32: 1501, 1938.
(149) WESTENBRINK, H. G. K. AND B. C. P. JANSEN. Acta. brevia. neerl. 8: 119, 1938.
(150) TAUBER, H. Proc. Soc. Exper. Biol. Med. 37: 541, 1937.
(151) KINNERSLEY, H. W. AND R. A. PETERS. J. Soc. Chem. Ind. 56: 934, 1937.
(152) v. EULER, H. AND R. VESTIN. Naturwiss, 25: 416, 1937.
(153) TAUBER, H. Science 86: 180, 1937.
(154) STERN, K. AND V. HOFER. Science 85: 483, 1937.
(155) STERN, K. AND V. HOFER. Enzymol. 3: 82, 1937.
(156) TAUBER, H. J. Am. Chem. Soc. 60: 730, 1938.
(157) WEIJLARD, J. AND H. TAUBER. J. Am. Chem. Soc. 60: 2263, 1938.
(158) WESTENBRINK, H. AND J. GOUDSMIT. Nature 142: 150, 1938.
(159) WESTENBRINK, H. AND J. GOUDSMIT. Enzymol. 5: 307, 1938.
(160) HENNESSY, D. J. AND L. R. CERECEDO. J. Am. Chem. Soc. 61: 179, 1939.

(161) Neuberg, C. and J. Hirsch. Biochem. Ztschr. 115: 282, 1921.
(162) Peters, R. A. and R. H. S. Thompson. Bioch. J. 28: 916, 1934.
(163) Peters, R. A., H. Rydin and R. H. S. Thompson. Bioch. J. 29: 53, 1935.
(164) Peters, R. A. Bioch. J. 30: 2206, 1936.
(165) Sherman, W. C. and C. A. Elvehjem. This Journal 117: 142, 1936.
(166) Krebs, H. and W. Johnson. Bioch. J. 31: 645, 1937.
(167) Weil-Malherbe, H. Bioch. J. 31: 2202, 1937.
(168) Lipmann, F. Skand. Arch. Physiol. 76: 255, 1937.
(169) Lipmann, F. Enzymol. 4: 65, 1937.
(170) Lipmann, F. Nature 138: 1097, 1936.
(171) Lippman, F. J. Am. Chem. Soc. 60: 2574, 1938.
(172) Williams, R. R. et al. J. Am. Chem. Soc. 57: 536, 1935.
(173) Lipschitz, M. A., V. R. Potter and C. A. Elvehjem. Bioch. J. 32: 474, 1938.
(174) Lipschitz, M. A., V. R. Potter and C. A. Elvehjem. J. Biol. Chem. 124: 147, 1938.
(175) Krebs, H. A. Klin. Wchnschr. 11: 1744, 1932.
(176) Warburg, O. and W. Christian. Biochem. Ztschr. 295: 261, 1938.
(177) Straub, F. Nature 141: 603, 1938.
(178) Warburg, O. and W. Christian. Naturwiss. 26: 201, 1938.
(179) Warburg, O. and W. Christian. Naturwiss. 26: 235, 1938.
(180) Warburg, O. and W. Christian. Biochem. Ztschr. 296: 294, 1938.
(181) Warburg, O., W. Christian and A. Griese. Biochem. Ztschr. 297: 417, 1938.
(182) Warburg, O. and W. Christian. Biochem. Ztschr. 298: 150, 1938.
(183) Needham, D. Biokhimiya 2: 489, 1937.
(184) v. Euler, H. et al. Ark. Kemi. Min. Geol. 12B: no. 24, 1936.
(185) Lohmann, K. Angew. Chem. 50: 97, 1937.
(186) Meyerhof, O. New England J. Med. 220: 49, 1939.
(187) Parnas, J. K. and P. Ostern. Bull. Soc. Chim. biol. 18: 1471, 1936.
(188) Kraut, H. and E. Bumm. Ztschr. physiol. Chem. 177: 125, 1928.
(189) Bumm, E., H. Appel and P. Couceiro. Ztschr. physiol. Chem. 220: 186, 1933.
(190) Kraut, H. and R. W. Nefflen. Ztschr. Physiol. Chem. 232: 270, 1935.
(191) Allison, F., S. Hoover and D. Burk. Science 78: 217, 1933.
(192) Hoover, S. and F. Allison. Trans. 3rd Int. Cong. Soil Sci. Oxford, 1935.
(193) Lohmann, K. Biochem. Ztschr. 254: 332, 1932.
(194) Cori, C. and G. T. Cori. Ann. Rev. Biochem. 1934.
(195) Morgan, E. J. and E. Friedmann. Biochem. J. 32: 733, 1938.
(196) Morgan, E. J. and E. Friedmann. Biochem. J. 32: 862, 1938.
(197) Szent-Gyorgyi, A. et al. Ztschr. physiol. Chem. 236: 1, 1935.
(198) Stare, F. J. and C. A. Baumann. Proc. Roy. Soc. B. 121: 338, 1936.
(199) Hopkins, F. G. Biochem. J. 15: 286, 1921.
(200) Green, D. E. and D. Richter. Biochem. J. 31: 596, 1937.
(201) Quastel, J. H. and W. J. Wooldridge. Biochem. J. 22: 687, 1928.
(202) Theorell, H. Biochem. Ztschr. 1275: 30, 1935.
(203) Warburg, O. Ergebn. Enzymf. 7: 210, 1938.

(204) THEORELL, H. Biochem. Ztschr. **278**: 263, 1935.
(205) KUHN, R. AND H. RUDY. Ber. Chem. Ges. **69**: 1974, 1936.
(206) WARBURG, O. AND W. CHRISTIAN. Biochem. Ztschr. **298**: 368, 1938.
(207) HAAS, E. Biochem. Ztschr. **298**: 378, 1938.
(208) BALL, E. Science **88**: 131, 1938.
(209) NEGELEIN, E. AND E. HAAS. Biochem. Ztschr. **282**: 206, 1935.
(210) NEGELEIN, E. AND H. J. WULFF. Biochem. Ztschr. **289**: 436, 1937.
(211) NEGELEIN, E. AND H. J. WULFF. Biochem. Ztschr. **290**: 445, 1937.
(212) NEGELEIN, E. AND H. J. WULFF. Biochem. Ztschr. **293**: 351, 1937.
(213) CORRAN, H. S. AND D. E. GREEN. Biochem. J. **32**: 2231, 1938.
(214) ELVEHJEM, C. A. ET AL. J. Biol. Chem. **123**: 137, 1938.
(215) SPIES, T. D., C. COOPER AND M. A. BLANKENHORN. J. A. M. A. **110**: 622, 1938.
(216) GYÖRGY, P., R. KUHN AND T. WAGNER-JAUREGG. Ztschr. physiol. Chem. **233**: 241, 1934.
(217) KUHN, R., P. GYÖRGY AND T. WAGNER-JAUREGG. Ber. Chem. Ges. **66**: 317, 1933.

LECTURES AND REVIEWS

MYRBÄCK, K. Cozymase. Ergebn. Enzymf. **2**: 139, 1933.
v. EULER, H. Cozymase. Ergebn. Physiol. **38**: 1, 1936.
KARRER, P. Über einige natürlich vorkommende, biochemisch bemerkenswerte Pigmente. Helv. chim. Acta. **19**: E 33, 1936.
WARBURG, O. AND W. CHRISTIAN. Pyridin der wasserstoffübertragende Bestandteil von Gärungsfermente. Helv. chim. Acta. **19**: E 79, 1936.
v. EULER, H. Die Coenzyme. Angew. Chem. **50**: 831, 1937.
MYRBÄCK, K. Cozymase. Tabul. biol. **14**: 110, 1937.
SCHLENK, F. Triphosphopyridine-nucleotid. Tabul. biol. **14**: 186, 1937.
KARRER, P. Les ferments. Bull. Soc. chim. belg. **46**: 351, 1937.
NEEDHAM, D. Coenzymes in muscle metabolism. Biokhimiya **2**: 489, 1937.
SCHLENK, F. Cocarboxylase. Tabul. biol. **14**: 193, 1937.
STERN, K. G. Recent experiments on the chemistry and mechanism of enzyme action. Enzymol. **5**: 190, 1938.
WARBURG, O. Chemische Konstitution von Fermenten. Ergebn. Enzymf. **7**: 210, 1938.

DENTAL CARIES

JOHN A. MARSHALL

University of California, College of Dentistry

A previous discussion of dental caries (Marshall, 1924) compared some of the historical viewpoints with the modern theories of its etiology. During the fourteen years which have elapsed since the publication of the review, a number of contributions have served to advance, in some degree, the ideas previously mentioned. By assessing arbitrary values to these later reports it is hoped that progress in research dealing with caries may be interpreted in terms of etiology, pathogenesis and prevention.

The original classification of the etiological factors followed for convenience an alphabetical arrangement, namely: A, anatomical; B, bacteriological; C, chemical; D, dietary; E, endocrine; F, failure in mouth hygiene; H, heredity. It will be noted that the first three, A, B, C, are essentially local in effect; the next two, D and E, are of systemic importance and the last two, F and H, comprise both local and systemic causes. The terms "direct and indirect," when applied to a classification of etiological factors in caries, have little meaning, for the reason that the relative importance of the seven sub-heads varies with age, environmental vicissitudes, habits, health and probably other, as yet undetermined, agents. Accordingly, each heading again will be discussed and the previous conclusions compared with current hypotheses. Since each factor has been intensively studied in many laboratories the viewpoint today has brought into sharper focus certain aspects which previously have been obscure.

It is a well known clinical observation that dental caries develops much faster in the deciduous teeth than in the second set. This inequality is due in part to dissimilarities in degree of calcification, and in the size and position of the teeth. Even slight irregularities of enamel surface favor plaque development and thus become susceptible areas for a primary carious lesion.

One of the greatest advances in the study of the pathogenesis of caries has been the experimental production of hypoplastic teeth. Areas which are deficient in formation not only are less dense but show also

surface inequalities or pits. The greater part of the research dealing with malnutrition, whether of dietary insufficiency, unbalanced mineral intake or endocrine dysfunction, is devoted to the study of the factors adversely affecting dental development.

Many writers, including Williams (1897), Walkoff (1915), Mummery (1923), and Marshall (1936), have confirmed the clinical and laboratory observation that there is scarcely a tooth in man or lower animal which does not present areas of incomplete formation. Dental literature contains many papers dealing with dental hypoplasia from the standpoint of pathological anatomy. However when a "defect" is so widespread, it ceases to have any diagnostic significance; on the contrary it becomes of histologic, rather than histopathologic interest. An individual six feet in height is as normal anatomically, as one who is five feet five inches in height; but at seven feet, or three feet in height, one postulates abnormality. So with dental tissues. But when an area of incomplete formation becomes a punctate depression, easily discernible with the unaided eye, or when deficiencies in cement substance impart an altered translucency to the enamel rods, either as white spots or as mottling, or when unclosed pits and fissures on incisal and occlusal surfaces are wide and deep enough to be recognized clinically, these defects present pathogenic significance.

One of the fundamental observations upon which a great deal of the subsequent research was built was a physico-chemical study conducted by Entin (1929). He suggested first, that caries might be regarded as a symptom of latent avitaminosis D; and second, that enamel and dentine possess properties of a semi-permeable membrane toward solutions of salts and dyes. Thus hypoplastic teeth are more permeable than "normal" ones in which calcification is dense and nearly completed. In an effort to increase calcium deposition in tooth structure Fish (1935) fed maximum doses of vitamin D plus calcium carbonate but found no measurable change in the calcium content of formed dentine in erupted teeth. As was emphasized in an earlier communication (Fish, 1932), caries begins in a permeable crevice or fault area. Upon the eruption of the tooth, the "fault" becomes filled with oral fluids containing food particles and microörganisms. If the lesion progresses slowly, a slight irritation arises in the dentine beneath the carious area. Hypercalcification results and thus a barrier is formed against the further invasion by caries. Beust (1931) confirmed Fish's views when he stated that the resistance which a tooth offers to caries is directly proportional to its hardness. The pathogenesis of caries

depends not only upon defective dental structures but also upon the susceptibility of the patient to the organisms of caries. But the questions of seasonal variations (Erpf, 1938) and of control of susceptibility remain unanswered, nor can the probable hereditary influences be assessed. In short, racial and hereditary factors require extensive study.

Karshan, Weiner, and Stofsky (1934), using specially prepared aqueous extracts of enamel and dentine, were unable to demonstrate important differences in carious and non-carious teeth in respect to hydrogen ion concentration, calcium, phosphorus and carbon dioxide. Experiments were made also on the determination of soluble protein and it was found that although the enamel presented only slight changes in carious and non-carious teeth, the dentine showed quite significant variations.

Applebaum and Adams (1938) report interesting data obtained from the use of the Grenz ray in the histopathologic study of carious teeth from rats. The several types of caries have been classified first, into those lesions found in fissures; second, in fractured enamel of molar cusps, and third, in fractures on the tip of the cusp. The Grenz ray brings out very clearly that fractures are adventitious, occurring both in carious and non-carious teeth and therefore not a part of the pathogenesis of caries. Decalcification is the important event. The evidence thus refutes the idea that in the rat caries develops only when the enamel is fractured.

Hodge and McKay (1933) by developing a new technic, have presented data which indicate that enamel comprising the outer third is greater in micro-hardness (Bierbaum hardness number) than that of the middle or inner thirds. This fact would have even greater significance if the teeth which were tested had been extracted from patients of known age.

Additional histological studies are offered by Cape and Kitchin (1930). Confirming Orban's views upon the eccentric deposition of lime salts in human enamel, they reported histologic observations made by studying sections illuminated with polarized light and with crossed nicol prisms. This field of research offers fascinating opportunities for further study, particularly of the different types of caries, both clinical and experimental.

Chemical determinations of "trace elements" in human teeth have identified strontium, barium, silver, lead, chromium, tin, and zinc and possibly traces of magnesium (Lowater and Murray, 1937). Although

teeth from individuals residing in and around London contain doubtful traces of fluorin, those from Maldon, using water having a relatively high percentage of fluorides, show the definite chemical reactions of these compounds. When fluorides are added to rats' diets these halogen derivatives are demonstrable in enamel, and such tissues may be classified as hypoplastic (Smith and Lantz, 1932). Mellanby's experiments (1928, 1930, 1932a, 1932b; Mellanby and Pattison, 1932) although criticized from other aspects (Shaw, 1932; Fish, 1932; Marshall, 1933) are acceptable at least in this connection, that defective dental calcification appears in teeth of experimental animals when diets are fed which are deficient in vitamin D and calcium and phosphorus.

In South Africa, as noted by Shaw (1932), rickets is practically unknown and if Mellanby's views are correct caries should be correspondingly low. But the opposite is true. In an examination of about 600 children only 6.6 per cent showed clinical evidence of sound teeth and 93.35 per cent presented one or more carious areas, the average for all being 4.84 carious teeth each.

In spite of these divergent views there is justification for the statement that of all the factors entering into the etiology of caries the imperviousness, or lack of it, of dental structures to certain solutions appears to be extremely important. Perhaps it was this thought that prompted Fish (1932) to define caries as a "saprophytic phenomenon occurring either in morphological crevices of development or acquired faults in the tooth surface" and to say that "the tooth is powerless to prevent the onset of caries though the progress of the disease may be retarded by vital reactions in the dentine."

Enright and collaborators (1932, 1934; Enright and Friesell, 1933; Enright, Friesell and Trescher, 1932) offer additional confirmation. They have summarized their experimental data by indicating that deficient diets and ensuing defective nutrition result in formation of teeth more susceptible to acids of fermentation than fully calcified teeth.

Comparative studies (Hess, Abramson and Lewis, 1934) on the incidence of caries in rachitic and non-rachitic children have shown that in rickets, the incidence in deciduous molars is 27.4 per cent, and is greater than that found in non-rachitic patients. It is suggested that the interference in calcium metabolism occurring in rickets is of greater etiological importance in caries of deciduous teeth than in caries of permanent teeth. This has been denied by Shelling and Anderson (1936), but confirmed by Eliot et al. (1934), who agree on several points with the group of research workers in Hess' laboratory, first, that

dental caries is found more often in children with a known history of rickets; second, that caries occurs more often in hypoplastic teeth; and third, that anti-rachitic therapy in infancy reduces the incidence of enamel hypoplasia.

From time to time there appear in the literature statements to the effect that the incidence of caries is higher in girls than in boys. Klein and Palmer (1938) conducted a survey on 4,416 children of school age, which indicated that caries incidence of girls very closely parallels that of boys. This would tend to support the findings of Cunningham (1934) at least in this respect, that disturbances of calcium metabolism incident to menstrual periods have little etiological effect in caries. In Cunningham's extensive survey physical examinations and dental charts of 11,000 women students were studied. She failed to find any significant relation between the number of damaged teeth and histories of illness, or menstrual function, or physical measurements. The count of damaged teeth, unfortunately, did not include the number of extracted teeth, an oversight which the author herself mentions in her report. Perhaps the admission of these figures would have led to a different summary of the research.

Another field for investigative effort, suggested by Bichlmayer (1935), is concerned with the badly broken down and carious tooth as a portal of entry for diseases such as tuberculosis. This matter has also received the attention of Snow (unpublished report) in his observations at Weimar Sanitarium. Although the possibility that infection may be carried through this channel cannot be denied, the preliminary data are too scattered to permit further statements.

To formulate an hypothesis which will reconcile the diverse opinions on the anatomical factors in the etiology of caries which have been so freely expressed, would be a task too laborious for this short review. An analysis of the facts however is possible. It becomes increasingly evident that structural weaknesses in enamel and dentine occur so frequently and have been produced so easily experimentally, that the relative density of teeth may be the controlling factor in immunity and susceptibility to caries.

The former concept of hypoplasia not only is inadequate but in certain respects is wrong. Attention is first invited to the dictum of G. V. Black and others to the effect that a tooth once erupted does not change in degree of calcification. Quite the contrary is the case. Both enamel and dentine in older people are much more difficult to cut with burr, stone or chisel than in young adults. The differences are even

greater between human permanent and deciduous teeth, and are less noticeable in similar teeth from monkey or dog. Dental density in humans usually increases with age. In the event of disease, either infectious or metabolic, this condensation of hard tissues may not develop.

Of equal importance is the next fact, now established in many laboratories, that both enamel and dentine possess properties of permeability provided that the dental pulp retains its vitality and is neither infected from without or within, nor inflamed from any mild but persistent trauma (severe trauma causes pulp necrosis). The passage of substances through these tissues is controlled by the density or degree of calcification in each tooth; the less dense the greater the permeability. If the mineral intake is unbalanced or if the nutritional factors become inadequate at any period from fetal life to twenty years of age, or if the individual contracts an exanthem, any or all of these circumstances are reflected in the degree of dental hypoplasia so produced (Marshall and Lucia, 1938). Dental permeability is greater when the nutritional factors are inadequate; thus not only are defectively calcified teeth more liable to be infected with caries but erupted teeth also change. It is possible that erupted teeth, instead of developing the usual condensation phenomena, retain their degree of permeability incident to their eruption, or, by a reverse reaction, become more permeable. Thus the inherent defense mechanism fails and rampant caries develops. Nor is this frank speculation, but is based, as will be shown later, on clinical and laboratory data.

The older idea, that the exanthemata of childhood were mainly responsible for the occurrence of hypoplastic teeth must be discarded. The knowledge of nutrition which has grown so rapidly during the last twenty years develops the premise that dental hypoplasia is a sequel, not only of certain infectious diseases but also of metabolic diseases in which category occur dietary deficiencies and endocrine dysfunctions. These facts also will be more fully developed in succeeding sections. Indeed hypoplasia of teeth can no longer be regarded as a disease; it is found so frequently that it must be classified as a minor anatomical variation.

Although the interest in the bacteriology of caries has been as keen as in its histo-pathology, the contributions have been less spectacular, but none the less important. Confirmation of the theories of W. D. Miller on the subject seem always to be the starting point, and in some instances unfortunately, the end point as well.

A relief from this reiteration is to be found in a paper by Dobbs (1932). In his research upon mucin plaques, it was demonstrated that these accumulations are more permeable to dextrose solutions than to salivary buffers. Carbohydrates in the mouth are hydrolyzed to various organic acids such as lactic acid, which may accumulate in concentrations great enough to decalcify enamel. It is at this point that Fosdick and Hansen (1936), and Fosdick, Hansen, and Messinger (1937) considered the possibility that acids, other than lactic, are produced as degradation products of carbohydrates. They suggest that pyruvic acid, although it has never been isolated as such from human saliva, is actively concerned in dental decalcification. However, the reductase activity of various oral flora transforms pyruvic acid to lactic acid in the presence of insoluble calcium salts.

Further research by Hansen, Fosdick, and Epple (1937) upon the formation of reducing sugars from soluble starch, has extended and confirmed their preliminary studies. In the initial stages of caries *B. acidophilus* by itself is incapable of exerting any decalcifying action on powdered enamel. This is the point which is at variance with Miller's theory. However, both in the initial and in the well developed lesion, when *B. acidophilus* is grown in symbiosis with *B. aerogenes* or with *Saccharomyces cerevisiae*, decalcification proceeds readily. This explains the observations of Mull, Bill and Kinny (1934), who have stated that *B. acidophilus* was not consistently present in caries nor consistently absent in immunity.

Bibby (1935), Bibby and Sedwick (1933) confirmed the theories of early experimenters, for example, Bodecker (1927, 1934), Bodecker and Applebaum (1932), Williams (1897) and others even earlier, who proved that decalcification of enamel is the initial process in caries rather than proteolysis of organic matrix of this tissue. This point had been questioned and needed re-investigation.

Jay and his colleagues (see bibliography, 1929, 1930, 1932, 1933, 1936, 1938) summarized the studies of the Michigan group by stating that:

1. Dental caries is unrelated to variation in dietary constituents nor can it be arrested by adding mineral and vitamin preparations. A diagnostic relationship exists however between oral lacto-bacilli and the amount of carbohydrate in the diet; thus, by restricting carbohydrate in diet, rampant caries can be effectively treated. One might be forced to quibble a bit in an effort to adjust the first statement with the latter; in one instance caries is unrelated to diet, and in the second, it can be controlled by diet. However, the inference is that dental

caries is unrelated to variation in dietary constituents except carbohydrates which, when restricted, play indirectly a part in reducing bacterial activity and consequently caries development. W. D. Miller said something to this effect in 1893.

The conclusions of the Michigan group lack confirmation in the experiments of Tucker (1932). He reports that aciduric streptococci were found in mouths of all children irrespective of the presence of caries. Furthermore, the ingestion of citrus fruits did not affect the bacterial count. The research indicates that *L. acidophilus* bears some relationship to carious cavities but is not an obligate factor in etiology. Boyd, Zentmire, and Drain (1933) confirm the observations of Tucker and extend them by concluding that children on a supervised dietary regimen exhibit no characteristic difference in the morphological nature of oral flora, from those whose diet is unrestricted. Furthermore Rosebury and Karshan (1931) report that a diet high in carbohydrates, but lacking entirely, or containing less than the physiological requirement in calcium and vitamins C and D, failed to produce caries, even though oral lactobacilli were fed in an adhesive gum tragacanth paste. But Etchells and Devereux (1933) were successful in an experiment in which pure cultures of aciduric organisms were introduced into a sterilized coarse ration. Control animals failed to develop caries, all others showed extensive lesions. Of course the experiments are not parallel except in so far as the bacteriological aspect is concerned, but on that point, at least, the success of Etchells and Devereux is convincing.

Hollander (1934), reporting for the Columbia University research group, concludes that the process of dental decay is associated with environmental factors, such as food retention and lacto-bacillary fermentation, and with physiological factors related to calcium and phosphorus metabolism. This summary confirmed previous reports from Columbia and other institutions.

Johnston et al. (1933, 1936) compared oral flora in rats with and without caries-like lesions and concluded that *Lacto-bacillus acidophilus* is not associated with the incidence of rat caries; in fact, this type of organism is normally present in all cases. In children a low count of oral bacteria was found alike in carious and non-carious mouths. Although they attach less importance to the presence of lacto-bacilli as an etiological factor of primary importance in caries, they suggest that teeth may develop a lowered resistance arising from nutritional disturbances and thus are more readily attacked by bacteria. This again infers that dental hypoplasia is a predisposing factor.

Rodriguez (1931) found that a high bacterial count of colonies per cc. of saliva apparently bears some relationship to age incidence of caries. Between 10 and 14 years there is a gradual rise, but the succeeding four years (14 to 18) show a slight decrease. In this connection Lyons (1935) studied cultures from carious teeth and presented data upon the percentage distribution of *L. acidophilus* in accordance with the age of the patient. During the first ten years this organism occurred in 84 per cent of the cases, and dropped to 66 per cent between 10 to 20 years; 49 per cent between 20 to 30 years; 20 per cent between 30 to 40 years; 14 per cent between 40 to 50 years; and 2 per cent from 51 on. Furthermore with advancing years there occurred a change in the type of organisms, in which flagellated bacilli and streptococci ultimately predominated. Rat caries was produced by mixing selected cultures of acid-forming bacteria with food.

In summarizing this section on the bacteriology of caries it is evident first, that both laboratory and clinical data suggest opposing points of view. One group maintains that the type of oral flora is the same in carious and non-carious mouths. The other group is equally emphatic in pointing out that aciduric organisms predominate in caries. It is possible that the suggestions of Hansen, Fosdick and Epple (1937) may serve to reconcile these two theories.

Although the chemical changes in saliva which were discussed in the earlier review have received scant attention, a few investigators have developed this field of research. Forbes (1931) in an excellent paper dealing with the theoretical aspects of the etiological factors, says that the neutralizing power of saliva against localized acid accumulations becomes a very important agent in the prevention of caries. It is likely that the concentration of alkaline salivary buffers is primarily determined by the alkali reserve of the blood. Since this in turn is partly a dietary problem it is necessary to differentiate between a diet that is potentially and one that is actually alkaline. It has been pointed out by Cole, Speer and Heyle (1929) that calcium and phosphorus retention is favored by a diet potentially alkaline; but on the other hand, retention is decreased when the diet is actually alkaline. One pauses to speculate upon the effect on the population of the promiscuous and unsupervised use of alkaline salts, which may increase actual alkalinity beyond the physiological optimum, and thus become one of the most insidious forms of self-medication. By following a diet which is potentially, rather than actually alkaline in character, blood alkali reserves

may be maintained. Recalling the statements of Forbes that the alkali reserves of the blood control the alkali reserve (neutralizing value) of saliva, it is evident that dietary supervision is a necessary part of the prophylaxis of caries.

The work of Eddy (1933, 1934) and Eddy, Heft, Rosenstock and Ralston (1933) dovetails very nicely with reports from other laboratories. They emphasized the fact that up to that time (1933) no worthwhile evidence had been presented to prove that sugar favors a constant production of lactic acid-forming organisms. The deleterious effect of sugar is found first, in the lowering of the blood phosphorus; second, in raising blood pH without changing the blood calcium; thus supporting the unpublished observations of Klein, Kruse and McCollum. The decrease in blood phosphorus results in a corresponding temporary decrease in salivary phosphorus. Therefore, salivary phosphorus can be controlled to some extent by dietary supervision. For example, it is lowered by rapid ingestion of sugar or other less readily digested carbohydrates, or proteins; it is unaffected by fats; and may be temporarily increased by inorganic phosphate or by dietary substances containing this element in correct nutritional proportions.

Determinations of hydrogen ion concentration still continue to interest some laboratories. However, there has never been a research described with sufficient accuracy in which the results have been other than negative, in so far as this characteristic of saliva is concerned (Eddy, 1934). Alteration of pH neither indicates immunity from, nor susceptibility to, caries; the variations are too small. Karshan (1931) and Krasnow (1936, 1938) reported the pH of blood and saliva, and determinations of calcium and phosphorus, protein, and carbon dioxide. They have pointed out that titratable alkalinity is a better criterion than the hydrogen ion concentration, because the pH values observed in caries-immune and caries-susceptible individuals either actually overlap or else differ so slightly as to be without significance, both from the diagnostic and etiologic standpoints. Perhaps other interpretations would be found if greater dilutions of standardized solutions were made. It has been shown that the one one-hundredth normal solutions give less accurate results than two-hundredth normal. The choice of indicators is equally important. Para-nitro-phenol far surpasses methyl orange for the determination of alkalinity; and phenophthalein titrated against sodium hydroxide two-hundredth normal is a very satisfactory method for determining acidity of saliva samples (Marshall, 1917, 1934).

It is suggested by Schnack (1932) that in considering the etiology of caries from the standpoint of the importance of saliva, it is necessary to divide the topic into three parts; dental caries is favored first, when the buffer action of the saliva is diminished; second, when imperceptible acid regurgitations occur from the stomach; third, by acid fermentation in localized areas around the teeth. White and Bunting (1935, 1936) tested findings reported by Grove and Grove (1935) and were unable to confirm the statements that the activity of dental caries is correlated with ammonia content and pH of saliva. Champion (1932) by producing a temporary alkalosis and later an acidosis by ingestion of different buffer salts, altered the carbonate-bicarbonate ratio of the blood and the pH of the urine. The saliva was unchanged.

Mull, Bill and Kinny (1934) determined titratable acidity in salivas of pregnant women both carious and immune, and showed that the differences were too slight to be of any diagnostic significance. If total neutralizing values had been computed these additional data might have been of considerable interest. Neither were they able to show that the values of calcium and inorganic phosphorus in pregnancy indicate any relation to the condition of the teeth. Hubbell (1933) and Hubbell and Bunting (1932) report similar results for calcium and phosphorus.

Boyd (1933, 1937) and Drain and Boyd (1935a, 1935b) examined over 100 children without finding any correlation between the incidence of caries and concentrations of calcium and phosphorus in blood and saliva. They suggest however the possibility of a relationship between the retention of calcium-phosphorus and immunity to caries. Becks and Wainwright (1934, 1938) and Becks and Simmonds (1935) reviewed and criticized extensively the technical procedures described by other investigators for the determinations of calcium and phosphorus in saliva. They report certain slight modifications in the methods of Halderson and Bergen and Kirk and Schmidt for calcium estimations, as well as a modified Bodansky method for phosphorus.

Quantitative determinations of salivary calcium, phosphorus, sugar, hydrogen ion concentration (potential acidity), and the total neutralizing power (the sum of actual acidity and alkalinity) have been reported by several laboratories. Probably one of the chief reasons for the glaring disagreement in results is the difficulty of securing representative samples of saliva. It has been emphasized many times that the innervation of the salivary glands is so complicated that diverse unpredictable stimuli may produce profound alteration in type and rate of

secretion. Until standard methods of collecting salivary samples can be devised and adhered to by all laboratories, the progress in this direction will be slow and barren of results.

The preceding sections dealing with the anatomical, bacteriological, and chemical factors in etiology have described in part the various effects observed by experimenters in their respective fields. The following section is restricted to a discussion of a few typical experiments selected to focus attention upon the effects arising from differences in mineral and vitamin intake as reflected in alterations in the osseous system, in dental development, in the incidence and in the treatment of caries. Researches on the effects of diet in relation to pathogenesis and prophylaxis of caries comprise first, laboratory studies in which various groups of animals were maintained on diets designed to favor either the development of caries-like lesions, or dental hypoplasia, or alterations in oral flora, particularly the elaboration of aciduric organisms; second, clinical studies on groups of children dealing with prophylaxis rather than pathogenesis. The majority of the experiments present perplexing questions for which the answers are too involved for clarity. In spite of the apparent disagreement one may follow a thread of concurrence through this dietary maze, even though it be composed unfortunately in some cases, of experiments futile in concept and lacking scientific accuracy.

The initial effect of a diet deficient in vitamin A, demonstrable in the incisal teeth of rats and guinea pigs, is atrophy of the ameloblasts and subsequently of the entire enamel organ. In rats, metaplasia and decalcification supervene, but in guinea pigs ossification results (Smith and Lantz, 1932; Wolbach and Howe, 1933). These changes in the developing enamel have their counterpart in dentine hypoplasia and in pulp metaplasia (Marshall, 1923, 1927a, 1927b, 1928, 1931, 1935).

More remarkable, however, are the observations of Orten, Burn and Smith (1937). Rats surviving about 365 days' restriction in amount of vitamin A develop odontomata in the region of the incisor teeth but not in the molar area. In some instances supernumerary incisors were formed and frequently the original incisors became distorted as a result of the tumor growth. Arnim and co-workers (1937a, 1937b, 1933, 1936) by restricting the mineral intake of rats produced resorption and rarefaction of the alveolar process, an increase of the fat of the bone marrow, irregularities in dentification and occasional caries. When the diet was restored to normal levels these changes were reversed to the

extent that osseous structure was rebuilt and secondary tubular dentine formed.

McCollum (1931) pointed out that a diet lacking each vitamin will produce deleterious effects upon dental tissues and that defective diets are responsible for hypoplasia or metaplasia after eruption of the tooth. Thus defective diets alter dental structures from within by changing the *character of the dentine. Although a low Cevitamic acid intake* produces startling changes in odontoblastic function, the emphasis which has been placed on this vitamin is not justifiable.

Rat caries said to be identical with human caries has been described by Blackberg and Berke (1932). Animals receiving a basal ration plus a therapeutic amount of vitamin D for 150 days were free from caries. Without vitamin D, one-third developed caries and when an excess of viosterol was administered, all animals had carious teeth. Histopathologic changes beginning with a low grade hyperemia progressed through the various degenerative stages to pulp necrosis. It was suggested *further by the authors that circulatory disturbances in the pulp may* lead to decalcification of dentine (internal resorption).

Lilly (1932, 1938; Lilly and Grace, 1932; Lilly and Wiley, 1934) stated that white rats on a rachitogenic diet developed florid rickets *with extreme bone and joint deformities. No dental caries was* observed. The same laboratory found no caries on high carbohydrate or high fat intake, but one hundred per cent caries in rats 125 days old fed on diets containing coarsely ground yellow cornmeal. The same diet became non-carious if the corn was cooked or if the coarse particles were further ground to pass a 60-mesh sieve. Rosebury and co-workers (1932, 1933, 1934) produced fissure caries in rats, histologically similar to fissure caries in man, but this lesion did not develop in animals younger than 100 days. Addition of calcium, phosphorus and cod liver oil to the deficient diet was without effect. They reported also that *they were unable to demonstrate any relation between incidence of* caries and the relative amounts of calcium and phosphorus in the blood and in the ash of teeth and bones. Similar observations were made by Bibby and Sedwick (1933). They compared the results produced on teeth by *feeding three types of experimental diets: first, a diet of yellow* corn, wheat gluten, calcium carbonate and sodium chloride gave the highest incidence of caries; second, a heterogeneous diet of hospital scraps resulted in relative immunity; third, the addition of cod liver oil to a deficient diet was without noticeable effect.

Shelling and Asher (1933) likewise report that the incidence of caries

is high in rats maintained on coarsely ground corn. It is infrequent on diets poor in this grain or in those composed of casein and starch, or of grains having particles finer and softer than corn. Although no relation could be determined between caries and sex in animals, the authors confirmed a fact mentioned earlier by Marshall (1927a) that the lesion is infrequent in young animals, especially those that have been maintained on a deficient diet for less than two months. Furthermore it is probable that there are etiological factors in rat caries which are inapplicable to man.

The clinical application of the experimental dietary studies has met in some instances with gratifying success; other reports have described only failure. Earnshaw (1936) presented statistical data in support of the thesis that caries is an index of malnutrition. A different viewpoint has been suggested by Osborne (1933) who has observed that the incidence of dental caries is as high in Australia as in other parts of the world, in spite of the fact that the diet in that locality is rich in vitamins. That the problem cannot be limited to a study of vitamins is indicated also by Day and Sedwick (1934a) who have stated that the administration of vitamin concentrates is without effect in reducing the incidence of caries.

As is pointed out by Ker (1936) it is doubtful that the term "odontoclasia" of Jones (1935; Jones and Crosland, 1936) is justified, for the reason that no proof has been advanced to show that this so-called condition is other than hypoplasia or rampant caries. It is said to be the result of a low alkali intake. The lack of convincing evidence in this study is unfortunate, because one is prone to accept with enthusiasm any advance in theories of etiology, pathogenesis or treatment, however vague the newer ideas may seem.

Some of the theories of Mellanby have been received coldly but others deserve more consideration. After recounting the experimental studies the conclusion is drawn, which has often been mentioned before, that dental hypoplasia results from deficient diets. One speculation, which is difficult to follow, is that the nature of the food during the developmental period determines whether the teeth later will show caries or pyorrhea. It is said that of the usual foodstuffs, oatmeal exhibits the greatest "decalcifying" power and white flour the least. An anticalcifying factor is postulated ("Toxamin"), which is assumed to interfere with the normal development of teeth. Although oatmeal and wheat germ contain more calcium and phosphorus than some of the other cereals, the fact that these elements are present as the relatively

insoluble inositolphosphate makes them less available (Bruce and Callow, 1932, 1934). A special committee of the Medical Research Council, of which Mrs. Mellanby was a member, in reviewing the experimental data presented by her from 1928 to 1936, has reported (1936) first, no definite evidence has been developed which indicates that diet exerts any effect upon the structure of permanent teeth; second, a high vitamin D intake during the years of dental formation and growth and before the full eruption of the permanent teeth, diminishes the incidence of caries, especially in premolars and second molars, but is of less effect on first molars (and presumably on incisors). Thus again experimental evidence has been advanced supporting the theory of the close parallelism of hypoplasia with caries (Marshall, 1938).

Kugelmass et al. (1934, 1936) state that children free from dental caries consistently show a dietary intake containing a preponderance of raw fruit and vegetables. This seems to be an important observation and a step in the right direction. It is quite possible that a study of caries-immune rather than of caries-susceptible individuals may disclose nutritional and endocrine balances exerting a greater regulatory effect than has been suspected.

Hess and Abramson (1931) question seriously the assumption made by some investigators that dental caries is a specific nutritional disorder. Similarly, Rosebury and Foley (1934) suggest that caries is not necessarily due to defective tooth structure. A survey of 319 children conducted by Brodsky (1933) failed to show any relationship between the level of calcium or phosphorus and the development or arrest of caries. Two of the statements are equally confusing, first, that "children with marked hypoplasia exhibited a low caries incidence," and second, that "ingestion of candy and sugar seemed not to be an appreciable factor." These observations are not in accord with those of other research workers in this field. McBeath (1932) compared dietary effects upon three different groups of children from three different orphanages and reached the following conclusions: first, no one dietary factor exerts a predominating influence over any other factor; second, control of caries by a directed regimen is possible.

The studies of Hanke (1933) have been reviewed extensively in the Journal of the American Medical Association (see: Hanke, review, 1934). It was pointed out in detail that conflicting statements in addition to inadequate data, both clinical and experimental, make this work seem a gesture rather than an accomplishment. His experiments, unfortunately, must be dismissed with the same comments as those which

found; only an increase in the body weight of the animal could be recorded. Gordon and Kuskin (1935) have studied the relation of glandular dysfunction to irregularities in time of dental eruption. In the normal child the beginning of teething, walking, and talking appears related to the thyroid and the anterior pituitary growth hormone. Delay in appearance of these attributes may serve as an indication of glandular abnormality, provided that other non-endocrine factors have been excluded. Reduction in blood calcium and a slight increase in the inorganic phosphate result when secretion of the parathyroid hormone is interfered with by para-thyroidectomy. In cases of hyperparathyroidism the bone changes are those of cystic fibrosis, but variations in tooth structure have not been reported.

The effects produced on calcium metabolism by glandular changes have been studied mainly from alterations in the skeletal system, but the dental aspect either has been ignored or else treated too superficially to be of value etiologically, pathologically, or therapeutically. Barrows' studies (1938) upon parathyroid extracts have shown that when these preparations are administered, the hyperparathyroidism so produced is characterized by an increase in blood acidity. As a result alkaline salts are dissolved out of the bone and osteoclasts phagocytose the salts (osteitis fibrosa). Thus osteoclastic activity is secondary to bone resorption. Continued injection eventually gives rise to an opposite reaction and "marble bone" is formed. The degenerative changes in the osseous system probably find a counterpart in hypoplastic dentogenesis.

Experiments of this kind stress the importance of adequate calcium utilization. All efforts directed toward an optimum diet are fruitless unless the various factors or circumstances contributing to transport and normal deposition of the several inorganic salts can be recognized. Unfortunately, the physiology as well as the pathological physiology of the endocrines is not fully understood and until further facts can be discovered, progress in this phase of etiology will be uncertain.

It was the opinion of the White House Conference on Child Welfare (1930) that no method of treatment has yet been devised which will either prevent or repair the ravages of dental caries. In spite of this pessimism, sufficient evidence is accumulating to warrant a distinctly optimistic attitude in regard to future methods of treatment.

It is well known that filling operations are n[illegible] satisfactory. A careful examination of 8,500 extracted tee[illegible] or

insoluble inositolphosphate makes them less available (Bruce and Callow, 1932, 1934). A special committee of the Medical Research Council, of which Mrs. Mellanby was a member, in reviewing the experimental data presented by her from 1928 to 1936, has reported (1936) first, no definite evidence has been developed which indicates that diet exerts any effect upon the structure of permanent teeth; second, a high vitamin D intake during the years of dental formation and growth and before the full eruption of the permanent teeth, diminishes the incidence of caries, especially in premolars and second molars, but is of less effect on first molars (and presumably on incisors). Thus again experimental evidence has been advanced supporting the theory of the close parallelism of hypoplasia with caries (Marshall, 1938).

Kugelmass et al. (1934, 1936) state that children free from dental caries consistently show a dietary intake containing a preponderance of raw fruit and vegetables. This seems to be an important observation and a step in the right direction. It is quite possible that a study of caries-immune rather than of caries-susceptible individuals may disclose nutritional and endocrine balances exerting a greater regulatory effect than has been suspected.

Hess and Abramson (1931) question seriously the assumption made by some investigators that dental caries is a specific nutritional disorder. Similarly, Rosebury and Foley (1934) suggest that caries is not necessarily due to defective tooth structure. A survey of 319 children conducted by Brodsky (1933) failed to show any relationship between the level of calcium or phosphorus and the development or arrest of caries. Two of the statements are equally confusing, first, that "children with marked hypoplasia exhibited a low caries incidence," and second, that "ingestion of candy and sugar seemed not to be an appreciable factor." These observations are not in accord with those of other research workers in this field. McBeath (1932) compared dietary effects upon three different groups of children from three different orphanages and reached the following conclusions: first, no one dietary factor exerts a predominating influence over any other factor; second, control of caries by a directed regimen is possible.

The studies of Hanke (1933) have been reviewed extensively in the Journal of the American Medical Association (see: Hanke, review, 1934). It was pointed out in detail that conflicting statements in addition to inadequate data, both clinical and experimental, make this work seem a gesture rather than an accomplishment. His experiments, unfortunately, must be dismissed with the same comments as those which

apply to Davis (1930), namely, that the clinical and laboratory data are insufficient and inadequate.

Clarke and Smith (1935) confirmed an observation which has been reported many times: that a limited mineral intake is reflected by retarded somatic growth. When the normal diet is substituted for the restricted one, growth is resumed.

Day (1934) and Day and Sedwick (1934b) conducted clinical studies on the influence which fat soluble vitamin A may exercise on the development of dental caries. To the ordinary diet, without attempting to regulate food intake, there were added six thousand U.S.P. units of vitamin A and fourteen hundred Steenbock units of vitamin D. Comparing one hundred and seventy-one controls with one hundred and forty-seven experimentals, the authors were of the opinion that the experimental group showed no reduction in caries incidence, as judged by average increase in percentage of infected teeth. An experimental period of fifteen months is probably inadequate for an age group of thirteen years. It will be recalled that the British Anthropometric Society presented figures to indicate that the greatest growth rate of the human occurs between birth and four years of age. This is also the time of greatest rate of dental development. The conclusions, therefore, of Day and Sedwick were really to be expected when one considers that the greater amount of dental development had already been accomplished at the beginning of the experiment.

Eddy (1934) discussing the Boyd, Drain, Nelson diet says it "improves tooth resistance to decay in test groups when contrasted with controls on regular orphanage regime." This diet increases the phosphorus intake from four to ten per cent, and all known vitamins, particularly A and D. Boyd, Drain and Stearns (1937) have greatly extended their earlier studies and have lately (1937) described a clinical experiment in which five subjects between three and six years of age were maintained on various dietary modifications for a total period of seven months. During this time frequent clinical examinations determined the extent and rate of decay, and caries was considered active when the exploring tine located an area of exposed dentin. The authors reported that a diet adequate in all respects except vitamin D reduced, but did not stop, the development of caries. When 600 units of vitamin D per day were added for nine weeks, the rate of caries development was arrested. It was emphasized that a considerable period must elapse after starting vitamin D therapy before caries control can be accomplished. Of more importance than this, however, is the state-

ment that almost complete arrest of caries may be obtained by dietary supervision.

Realizing the fallacy of drawing comparisons too closely between animal experimentation and clinical experience, one may yet be justified in pointing out that the present situation in the epidemiology of human caries may be the indirect result of deficient diets and particularly those classified by McCollum as "borderline." Some support is given this premise when it is recalled that laboratory experiments produced lesions resembling caries, when animals were maintained on deficient diets for relatively long experimental periods (with rats, six to eight months). However, with second and third generation animals so controlled, this result is more easily gained (Marshall, 1933). Perhaps it is not too far-fetched to suggest that the human race today is suffering from dietary insufficiencies of former generations. Caries is only one of the least of these unfortunate results.

In the field of endocrinology speculation has far outdistanced accomplishment. While the importance of normal glandular function for calcium utilization is readily recognized, it must be pointed out again that the influence of the ductless glands is probably less direct than other factors concerned in the etiology. Glandular dysfunction may be reflected in terms of dental hypoplasia, agenesis, malformation or malposition. Attempts to correct dystrophies in either osseous or dental systems by the administration of various biologic products have not been sufficiently studied to permit definite conclusions. Only a few scattered cases have been reported in which any known improvement in bone formation has been observed. Furthermore, there is less corroborating evidence dealing with alveolar bone than with other parts of the osseous structure. Thus it is evident that after eruption of teeth, endocrine dysfunction may contribute to dental malposition but not to dental malformation.

Jung and Skillen (1929) working with thyro-parathyroidectomized rats, described changes in enamel epithelium of an atrophic nature, confirming and extending Erdheim's experiments but contradicting Gies' comments (Gies and collaborators, 1930). Schour and Van Dyke (1932a, 1932b, 1933) have reported that hypophysectomized rats showed a progressively slower and slower rate of eruption of incisors. Eventually eruption ceased. Upon administration of pituitary growth-promoting hormone, there was a slight increase in the rate of eruption. When given to normal animals, however, no effect on eruption rate was

found; only an increase in the body weight of the animal could be recorded. Gordon and Kuskin (1935) have studied the relation of glandular dysfunction to irregularities in time of dental eruption. In the normal child the beginning of teething, walking, and talking appears related to the thyroid and the anterior pituitary growth hormone. Delay in appearance of these attributes may serve as an indication of glandular abnormality, provided that other non-endocrine factors have been excluded. Reduction in blood calcium and a slight increase in the inorganic phosphate result when secretion of the parathyroid hormone is interfered with by para-thyroidectomy. In cases of hyperparathyroidism the bone changes are those of cystic fibrosis, but variations in tooth structure have not been reported.

The effects produced on calcium metabolism by glandular changes have been studied mainly from alterations in the skeletal system, but the dental aspect either has been ignored or else treated too superficially to be of value etiologically, pathologically, or therapeutically. Barrows' studies (1938) upon parathyroid extracts have shown that when these preparations are administered, the hyperparathyroidism so produced is characterized by an increase in blood acidity. As a result alkaline salts are dissolved out of the bone and osteoclasts phagocytose the salts (osteitis fibrosa). Thus osteoclastic activity is secondary to bone resorption. Continued injection eventually gives rise to an opposite reaction and "marble bone" is formed. The degenerative changes in the osseous system probably find a counterpart in hypoplastic dentogenesis.

Experiments of this kind stress the importance of adequate calcium utilization. All efforts directed toward an optimum diet are fruitless unless the various factors or circumstances contributing to transport and normal deposition of the several inorganic salts can be recognized. Unfortunately, the physiology as well as the pathological physiology of the endocrines is not fully understood and until further facts can be discovered, progress in this phase of etiology will be uncertain.

It was the opinion of the White House Conference on Child Welfare (1930) that no method of treatment has yet been devised which will either prevent or repair the ravages of dental caries. In spite of this pessimism, sufficient evidence is accumulating to warrant a distinctly optimistic attitude in regard to future methods of treatment.

It is well known that filling operations are not entirely satisfactory. A careful examination of 8,500 extracted teeth showed that 5,435 or

about 60 per cent were carious (Marshall, 1937). Of these there were 1,746 or about 32 per cent which showed secondary caries under or around the margins of fillings. It is this latter figure which is more significant than first appears. The factors contributing to the incidence of secondary caries comprise all those previously mentioned in the etiology and in addition include inherent difficulties in operating, in sterilizing the cavities preparatory to filling and the undesirable physical and chemical properties of the filling materials. McLean (1935) is accurate when he says that fillings of a given classification are not uniform in chemical and physical qualities and therefore do not always prove satisfactory from a standpoint of caries prevention.

Brekhus and Armstrong (1936) suggest that cod liver oil, orange juice and tooth brushes alike have failed to prevent caries. Furthermore, "When human beings were using their teeth and were dependent upon them for existence, nature furnished them unstintingly. There was no caries and no pyorrhea." It is hardly necessary to point out in this connection that anthropologists and Egyptologists overwhelmingly refute this inference. The statistics of caries presented by Brekhus and Armstrong show only one part of the whole problem. To make their data of value it would be necessary to examine the same individuals after five years, ten years, and fifteen years. The figures would then present a true perspective which now is lacking. Suk (1936) indicts the plan of oral hygiene by saying that the races with the worst teeth do *the most tooth brushing and mouth washing. He could have done* better if he had added that these same races are nearer to dietary insufficiencies than other races.

Kugelmass (1936) suggests a ten point plan for the clinical control of caries in which are included not only the recognized regimen of public health control, immunization, and physical measurements, but also dietary supervision and periodic dental examination. Stone (1936) invites the physician to coöperate more closely with the dentist in an effort to control early ravages of caries in deciduous teeth.

Klein (1938a, 1938b) classifies for convenience teeth as decayed, missing, filled, and presents tabular data in support of the thesis that dental caries in childhood constitutes a health problem of major importance. The plan for providing more adequate dental care is based on a proposal that all dental defects in permanent teeth be treated in children of five to six years of age who attend the first grade in school, and also, each succeeding group of first grade children. As these students mature, the previous operations may be checked or repeated and

any new cavities filled. The plan requires the full coöperation of the community, the school authorities and the dental profession alike.

The rôle of dentifrices, tooth pastes, mouth washes, deodorants and in general all preparations or solutions either of true or alleged antiseptic value is insignificant in therapy of caries. None is effective, for rapid dilution by saliva renders them valueless except for lavage or momentary stimulus to glandular action. But a new study on caries prevention is being made in several localities. Ammoniacal silver nitrate is applied on small areas not yet carious, but so situated on tooth surfaces that they lie in the susceptible zones, not easily accessible for the tooth brush. This plan has many advantages but the results of the treatment cannot be assessed for several years (Prime, 1936). Some preparations, mentioned as antiseptic agents for renal infections, have also been suggested as suitable for oral therapy. Although other laboratories have had less success than Bunting, Hadley, and Hard (1930, 1936; also Bunting, Delves and Hard, 1930), these workers regard hexylresorcinol as the most effective bactericide in the prophylactic treatment of caries. The supplementary dietary measures provide for adequate proportions of milk and green vegetables, and the reduction of sugar consumption to a physiological minimum.

After an exhaustive review of the literature (1936) the Council of Dental Therapeutics was forced to conclude that there is no evidence available to warrant the use of calcium-phosphorus compounds for the prevention and treatment of caries. The claims of the manufacturers and of the various essayists are unfounded and such products lack a proven value as therapeutic agents. East (1938) reports satisfactory results based on follow-up data on dietary studies conducted on children in institutions. By following the suggestions of Boyd and Drain, caries incidence was lowered. Privitera (1938) also confirms these results.

Caries therapy has advanced during the last few years, first, by focussing public attention on the seriousness of the problem; second, by devising a quick and easy method of prophylaxis of susceptible areas; third, by instituting dietary supervision whenever possible; fourth, by improving the physical properties of the filling materials. The high incidence of this unfortunate disease is not generally understood, nor its sequelae. Any plan for intensive coöperative research should include studies in all of the various factors which have been discussed, bearing in mind that agencies which favor normal dental development demand intensive investigation.

SUMMARY

Regretfully one is forced to acknowledge that most of the factors which have been tested have been confined to selected groups of individuals. Suppose a comparison of rachitic and non-rachitic groups indicated a correlation of caries and hypoplasia in the first set of persons but not in the second. What about the other etiological factors? In some of the experiments it appears that the errors produced by "random sampling" have not been considered nor the different hereditary influences assessed.

It is evident that the contributions within the last fourteen years to the etiology, pathogenesis, prognosis and therapy of caries are of immense value from the standpoint of prevention. Anatomical defects in teeth are of histologic as well as pathologic importance. Therefore the secondary factors—dietary, endocrine, hereditary—contributing to the formation of poorly calcified teeth assume greater importance than in the past. Progress also in the solution of other problems relating to local conditions (bacterial, chemical) is of such importance (the pyruvic acid hypothesis, for instance) that the theories of Miller again must be revised to include the suggestions of Hansen, Fosdick et al. upon the local elaboration of acids other than lactic acid by definite bacterial symbiotic relationships hitherto unsuspected.

Caries may be treated now more successfully than ever before; not only has dietary supervision reduced the number of carious teeth but even through the periods of pregnancy and lactation the lesions may be controlled to some extent. Both mother and child respond to the regimen. In addition the wider application of ammoniacal silver nitrate solutions, although delayed twenty years, promises much in the control of the earlier stages of dental decay.

In spite of the advance in the therapeutics of caries, the etiology remains an enigma. No explanation of immunity to caries has been advanced; the lack of decay among the inhabitants of the island Tristan Da Cunha in spite of the high carbohydrate content of the diet, is certainly an incontrovertible argument against the theories now accepted. Rampant caries occurs and disappears without aid or hindrance of physician or dentist. These are only a few of the puzzles awaiting solution. Satisfactory answers must be found for the following queries:

1. Why does caries show a definite age incidence, for example, at adolescence? One suggestion is that the atrophy of the thymus gland at puberty, coincidental with the activity of the sex glands, may tem-

porarily upset the calcium-phosphorus balance and presumably also affect adversely the phenomena of dentogenesis.

2. What relation exists between the amount, content and acidity of salivary secretion, and caries? No reports on hydrogen ion concentration are satisfactory and too much confusion exists in relation to buffer value.

3. What are the relationships between blood calcium and blood phosphorus and the corresponding salivary percentages? The answer "dietary deficiencies and endocrine unbalance," is too vague.

4. Is there any relation between salivary mucin and rapid plaque formation? None has been proved.

5. How may malnutrition, both dietary and endocrine, be studied from a more comprehensive dental viewpoint?

6. What new methods of treatment, such as filling, sterilization of initial lesions, or diet regime may be devised for further control of this disease?

Caries affects 90 per cent of the population of the civilized world; it requires research in the exact and experimental sciences, and its conquest is a challenge to the physicist, chemist, dentist, anatomist, bacteriologist, physiologist, physician, pathologist and biochemist.

REFERENCES

To include in the references all the articles which have been published during the last fourteen years would serve no other purpose than to perpetuate to some degree many reviews, comments and opinions which lack scientific background. Such a reiteration is unnecessary. On the other hand no deliberate omission of a research is intended, provided that the experimental data are sufficiently adequate to justify whatever conclusions the author may present.

Applebaum, E. and T. H. Adams. J. Dent. Res. **17**: 95, 1938.
Arnim, S. S. Internat. J. Orthod. **23**: 1045, 1937a.
Arnim, S. S., S. S. Abele and E. H. Pitney. J. Am. Dent. Assn. **24**: 478, 1937b.
Arnim, S. S., M. F. Clarke and B. G. Anderson. Yale J. Biol. Med. **9**: 117, 1936.
Arnim, S. S., M. F. Clarke, B. G. Anderson and A. H. Smith. J. Biol. Chem. **100**: Proc. viii, 1933.
Barrows, R. B. Am. J. Anat. **62**: 237, 1938.
Becks, H. and N. Simmonds. J. Am. Dent. Assn. **22**: 1724, 1935.
Becks, H. and W. W. Wainwright. J. Dent. Res. **14**: 387, 425, 1934. J. Dent. Res. **17**: 197, 217, 1938.
Beust, T. B. J. Dent. Res. **11**: 619, 1931.
Bibby, B. G. J. Am. Dent. Assn. **22**: 222, 1935.
Bibby, B. G. and H. J. Sedwick. J. Dent. Res. **13**: 429, 1933.
Bichlmayr. Deutsch. Tuberk. Bl. **9**: 163, 1935.

BLACKBERG, S. N. AND J. D. BERKE. J. Dent. Res. 12: 609, 1932.
BÖDECKER, C. F. Dent. Cosmos 69: 987, 1927.
Bull. N. Y. Acad. Med. 10: 553, 1934.
BÖDECKER, C. F. AND E. APPLEBAUM. Dent. Cosmos 74: 335, 1932.
BOYD, J. D., C. L. DRAIN AND G. STERNS. J. Biol. Chem. 103: 327, 1933.
Proc. Soc. Exper. Biol. Med. 36: 645, 1937.
BOYD, J. D., Z. ZENTMIRE AND C. L. DRAIN. J. Dent. Res. 13: 443, 1933.
BREKHUS, P. J. AND W. D. ARMSTRONG. J. Am. Dent. Assn. 23: 1459, 1936.
BRODSKY, R. H. J. Am. Dent. Assn. 20: 1440, 1933.
BRUCE, H. M. AND R. K. CALLOW. Brit. Med. J. 2: 172, 1932.
Biochem. J. 28: 517, 1934.
BUNTING, R. W., E. A. DELVES AND D. G. HARD. Proc. Exper. Biol. Med. 27: 426, 1930.
BUNTING, R. W., F. P. HADLEY AND D. G. HARD. Am. J. Dis. Child. 40: 536, 1930.
J. Am. Dent. Assn. 23: 1369, 1936.
CAPE, A. T. AND P. C. KITCHIN. J. Am. Dent. Assn. 17: 193, 1930.
CHAMPION, A. H. R. Brit. Dent. J. 53: 209, 1932.
CLARKE, M. F. AND A. H. SMITH. Am. J. Physiol. 112: 286, 1935.
COLE, V. B., J. H. SPEER AND F. W. HEYLE. J. Am. Phar. Assn. 18: 107, 1929.
Council of Dental Therapeutics. J. Am. Dent. Assn. 23: 1369, 1936.
CUNNINGHAM, R. L. J. Dent. Res. 14: 439, 1934.
DAVIS, S. L. Report Bureau Investigation, J. A. M. A. 94: 1163, 1930.
DAY, C. D. M. Dent. Cosmos 76: 683, 1934.
DAY, C. D. M. AND H. J. SEDWICK. J. Nutrition 8: 309, 1934a.
J. Dent. Res. 14: 214, 1934b.
DOBBS, E. C. J. Dent. Res. 12: 455, 853, 1932.
DRAIN, D. L. AND J. D. BOYD. J. Am. Dent. Assn. 22: 155, 1935a.
J. Am. Dietetics 10: 471, 1935b.
EARNSHAW, P. A. Med. J. Australia 2: 4, 1936.
EAST, B. R. Am. J. Public Health 28: 72, 1938.
EDDY, W. H. J. Dent. Res. 13: 54, 1933.
J. N. Y. Acad. Dent. 1: 15, 1931.
EDDY, W. H., H. L. HEFT, S. ROSENSTOCK AND R. RALSTON. J. Dent. Res. 13: 511, 1933.
ELIOT, M. M., S. P. SOUTHER, B. G. ANDERSON AND S. S. ARNIM. Am. J. Dis. Child. 48: 713, 1934.
ENRIGHT, J. J. J. Dent. Res. 12: 571, 1932.
ENRIGHT, J. J. AND H. E. FRIESELL. J. Am. Dent. Assn. 20: 897, 1933.
ENRIGHT, J. J., H. E. FRIESELL AND M. O. TRESCHER. J. Dent. Res. 12: 759, 1932.
ENTIN, D. A. Ztschr. f. Stomatol. 27: 239, 1929.
ERPF, S. F. J. Am. Dent. Assn. 25: 693, 1938.
ETCHELLS, J. L. AND E. D. DEVEREUX. Proc. Soc. Exper. Biol. Med. 30: 1042, 1933.
FISH, E. W. Brit. Med. J. 2: 747, 1932.
J. Physiol. 84: 272, 1935.
FORBES, J. C. J. Dent. Res. 11: 591, 1931.

Fosdick, L. S. and H. L. Hansen. J. Am. Dent. Assn. **23:** 401, 1936.
Fosdick, L. S., H. L. Hansen and G. D. Messinger. J. Am. Dent. Assn. **24:** 1445, 1937.
Gies, W. J. and collaborators. J. Dent. Res. **10:** 215, 1930.
Gordon, M. B. and L. Kuskin. J. Pediat. **7:** 89, 1935.
Grove, C. J. and C. T. Grove. J. Am. Dent. Assn. **22:** 247, 1935.
Hanke, M. T. Diet and dental health. Univ. Chicago Press, 1933.
His book reviewed. J. Am. Med. Assn. **102:** 2136, 1934.
Hansen, H. L., L. S. Fosdick and S. F. Epple. J. Am. Dent. Assn. **24:** 1611, 1937.
Hess, A. F. and H. Abramson. Dent. Cosmos **73:** 849, 1931.
Hess, A. F., H. Abramson and J. M. Lewis. Am. J. Dis. Child. **47:** 477, 1934.
Hodge, H. C. and H. McKay. J. Am. Dent. Assn. **20:** 227, 1933.
Hollander, F. J. Dent. Res. **14:** 303, 1934.
Hubbell, R. B. Am. J. Physiol. **105:** 436, 1933.
Hubbell, R. B. and R. W. Bunting. Nutrition **5:** 599, 1932.
Jay, P. Am. J. Public Health **28:** 759, 1938.
Jay, P., M. Crowley and R. W. Bunting. J. Am. Dent. Assn. **19:** 265, 1932.
Jay, P., M. Crowley, F. P. Hadley and R. W. Bunting. J. Am. Dent. Assn. **20:** 2130, 1933.
Jay, P. and A. Esser. J. Am. Dent. Assn. **17:** 1117, 1930.
Jay, P., F. P. Hadley and R. W. Bunting. J. Am. Dent. Assn. **23:** 846, 1936.
Jay, P. and R. S. Voorhees. J. Am. Dent. Assn. **16:** 2054, 1929.
Johnston, M. M., M. J. Kaaka and M. C. Agnew. J. Am. Dent. Assn. **20:** 1777, 1933.
Johnston, M. M., C. H. M. Williams, P. G. Anderson, T. G. H. Drake, F. F. Tisdall and M. J. Kaaka. J. Am. Dent. Assn. **23:** 1493, 1936.
Jones, M. R. Dent. Cosmos **77:** 535, 651, 747, 1935.
Jones, M. R. and G. N. Crosland. U. S. Navy Med. Bull. **34:** 181, 1936.
Jung, F. T. and W. G. Skillen. Proc. Soc. Exper. Biol. Med. **26:** 598, 1929.
Karshan, M. J. Dent. Res. **11:** 573, 1931.
Karshan, M., R. Weiner and N. Stofsky. J. Dent. Res. **14:** 445, 1934.
Ker, A. J. J. Am. Dent. Assn. **23:** 17, 1936.
Klein, H. Pub. Health Bull. no. 239, 1938a.
Pub. Health Bull. **53:** 75, 1938b.
Klein, H., H. D. Kruse and E. V. McCollum. Unpublished report.
Klein, H. and C. E. Palmer. Pub. Health Report no. 1986, 1938.
Krasnow, F. J. Dent. Res. **15:** 366, 1936.
Am. J. Pub. Health **28:** 325, 1938.
Kugelmass, L. N. Internat. J. Orthod. **22:** 490, 1936.
Kugelmass, L. N., K. T. Newton and C. F. Bödecker. J. Am. Dent. Assn. **21:** 110, 1934.
Lilly, C. A. J. Nutrition **5:** 175, 1932.
Proc. Soc. Exper. Biol. Med. **38:** 398, 1938.
Lilly, C. A. and J. D. Grace. Proc. Soc. Exper. Biol. Med. **30:** 176, 1932.
Lilly, C. A. and L. Wiley. J. Nutrition **7:** 463, 1934.
Lowater, F. and M. M. Murray. Biochem. J. **31:** 837, 1937.
Lyons, D. C. J. Am. Dent. Assn. **22:** 409, 1935.

MARSHALL, J. A. Am. J. Physiol. 43: 212, 1917.
La Rev. d. Stomatol. 25: 579, 1923.
Physiol. Rev. 4: 564, 1924.
J. Am. Dent. Assn. 14: 3, 1927a.
J. Am. Dent. Assn. 14: 2207, 1927b.
J. Am. Dent. Assn. 15: 295, 1928.
Internat. J. Orthod. 17: 527, 1931.
Pac. Dent. Gaz. 40: 205, 1932.
Dent. Cosmos 75: 773, 1933.
Bull. Colo. St. Dent. Assn. 12: 11, 1934.
J. Am. Dent. Assn. 22: 1545, 1935.
J. Am. Dent. Assn. 23: 2074, 1936.
J. So. Calif. St. Dent. Assn. 4: 1462, 1937.
J. So. Calif. St. Dent. Assn. 5: 332, 1938.
MARSHALL, J. A. AND S. P. LUCIA. J. Am. Dent. Assn. 25: 943, 1938.
MCBEATH, E. C. J. Dent. Res. 12: 723, 1932.
MCCOLLUM, E. V. J. Dent. Res. 11: 553, 1931.
MCLEAN, D. W. Hygeia 13: 326, 1935.
Medical Research Council Report. Lancet 2: 280, 1936.
MELLANBY, M. Brit. Med. J. 2: 1079, 1928.
Brit. Med. J. 1: 292, 1930.
Brit. Med. J. 2: 749, 1932a.
Dental Survey 8: 53, 1932b.
MELLANBY, M. AND C. L. PATTISON. Brit. Med. J. 1: 507, 1932.
MULL, J. W., A. H. BILL AND F. M. KINNEY. Am. J. Obst. and Gynec. 27: 679, 1934.
MUMMERY, J. H. Structure of the teeth in relation to dental disease. 1923, London.
ORTEN, A. W., C. G. BURN AND A. H. SMITH. Proc. Soc. Exper. Biol. Med. 36: 82, 1937.
OSBORNE, W. A. Nutrition Abstract 2: 841, 1933.
PRIME, J. M. J. Am. Dent. Assn. 23: 785, 1936.
PRIVITERA, A. T. Arch. Pediat. 55: 206, 1938.
RODRIGUEZ, F. E. J. Am. Dent. Assn. 18: 2118, 1931.
ROSEBURY, T. AND G. FOLEY. J. Dent. Res. 14: 359, 1934.
ROSEBURY, T. AND M. KARSHAN. J. Dent. Res. 11: 121, 1931.
ROSEBURY, T., M. KARSHAN AND G. FOLEY. J. Dent. Res. 12: 464, 1932.
J. Dent. Res. 13: 379, 1933.
J. Am. Dent. Assn. 21: 1599, 1934.
SCHNACK, A. G. J. Am. Dent. Assn. 19: 62, 1932.
SCHOUR, I. AND H. B. VAN DYKE. Proc. Soc. Exper. Biol. Med. 29: 378, 1932a.
Am. J. Anat. 50: 397, 1932b.
Proc. Soc. Exper. Biol. Med. 50-51: 397, 1933.
SHAW, J. C. M. So. African Med. J. 6: 147, 1932.
SHELLING, D. H. AND G. M. ANDERSON. J. Am. Dent. Assn. 23: 840, 1936.
SHELLING, D. H. AND D. E. ASHER. J. Dent. Res. 13: 363, 1933.
SMITH, M. C. AND E. M. LANTZ. J. Dent. Res. 12: 556, 1932.
SNOW, G. W. Unpublished report.

Stone, J. B. South. Med. J. 29: 731, 1936.
Suk, V. Lancet 2: 470, 1936.
Tucker, W. H. J. Infectious Dis. 51: 444, 1932.
Walkhoff, O. Die Erdsalze in ihrer Bedeutung für die Zahncaries. 1915, Berlin, Herman Meusser.
White, J. and R. W. Bunting. J. Am. Dent. Assn. 22: 468, 1935. Am. J. Physiol. 117: 529, 1936.
Williams, J. L. Dent. Cosmos 49: 55, 1897.
Wolbach, S. B. and P. R. Howe. Am. J. Path. 9: 275, 1933.

THE MINERAL METABOLISM OF NORMAL INFANTS

GENEVIEVE STEARNS

Department of Pediatrics, State University of Iowa, Iowa City

The inorganic elements of the body may be classified according to their manner of use. Calcium is deposited chiefly in the skeleton, phosphorus and magnesium occur both in skeleton and in the soft tissues. Chloride, sodium and potassium are associated chiefly with the amount of body water and its distribution between intra- and extracellular fluids. Other minerals, as iron and iodine, vary in amount with the metabolism of the special tissues with which they are concerned.

Knowledge of the metabolism of inorganic elements in infancy requires primarily a study of changes in inorganic composition of the body during a period of very rapid growth, and is concerned equally with changes in composition occurring both before and after birth. The chemical composition of the fetus at different stages of gestation has been studied by direct analysis. Changes in composition of the growing infant must be studied by indirect methods, the customary procedure being that of Lindberg, who estimated from metabolic studies the quantity of substance retained during the time required for the infant to gain 100 grams in weight. This method of study is open to several obvious errorrs, but nevertheless is of considerable value, particularly in comparing the effects of dissimilar dietary regimens on body composition.

Moulton studied changes in body composition of mammals, including humans, during growth and observed that, if calculated on a fat-free basis, changes in nitrogen, ash and water conformed to definite laws, the water content varying inversely with the nitrogen and ash. Yannet and Darrow have brought forward evidence that the changes in composition of separate tissues during growth conform to the law of constant differential growth ratio as defined by Huxley; Hamilton has applied the same law to the changes in total water content of the growing human fetus. If the law holds for body water, it presumably holds also for the changes in body composition in regard to other substances, particularly those like calcium, which are added to the body by simple accretion and utilized almost entirely by one type of tissue.

Calcium. As over 98 per cent of body calcium is found in bone, a discussion of calcium metabolism is a discussion of skeletal growth and mineralization. Analyses of the calcium content of fetuses of various ages are numerous and show that the relative, as well as the absolute body content of calcium increases steadily throughout the fetal period (chart 1) so that at birth, the body of the normal infant of 3000 grams weight contains about 25 grams of calcium. If the mother's body is severely depleted of calcium during pregnancy, as in osteomalacia, the body of the infant may contain much less than the normal amount of

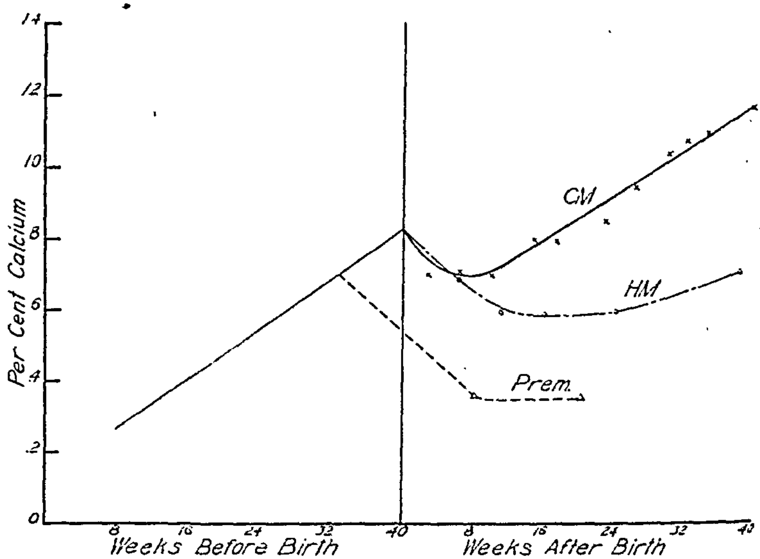


Chart 1. Changes in relative calcium content of the fetus and infant. The regression line of calcium content of fetus is drawn from data in the literature. *CM*—infants fed cow's milk; *HM*—infants fed human milk; *Prem.*—prematurely born infants fed human milk.

calcium and fetal rickets may occur. The evidence as to the effect of less severe calcium undernutrition of the mother on the fetal content of calcium is more conflicting. Booher and Hansmann could find no difference in the percentage of calcium in fetal bone when the mothers' diets varied widely in calcium content. On the other hand, Toverud has reported increasing incidence of soft skull and early rickets in infants born of mothers whose diets had been low in calcium, and Sontag reports roentgen-ray evidence of poorer bone structure in such infants. Increasing numbers of reports of rickets in very young infants are found in the literature. According to Toverud, the incidence of prematurity also

increases with poor maternal nutrition. Diets high in calcium, phosphorus and vitamin D during pregnancy are known to result in increased serum calcium and inorganic phosphorus levels of the fetus and are said to result in fetuses with greater than average density of bone. The possibility of an effect of diet in pregnancy on bone size of the infant cannot be disregarded. Undoubtedly the fetus grows at the expense of the maternal tissues, but evidence seems to be accumulating that this mechanism acts only within limits, and the maternal diet, especially in its calcium and vitamin D content, exerts a real effect on the skeletal development of the fetus.

Assuming that the calcium content of the body of the infant is within normal limits at birth, the amount of calcium retained daily by the infant will depend largely on the daily calcium intake and the quantity of vitamin D ingested or received through ultra-violet irradiation. Because of space limitations, discussion will be confined chiefly to the extremes of intake permitted by customary dietary regimens—human milk without added vitamin D and undiluted cow's milk with an added source of vitamin D.

The composition of human milk varies widely among individual women and is dependent, at least in some measure, on the adequacy of the diet. The reported values for calcium content range from 68 to 717 mgm. to the liter, with an average value of about 300 mgm. to the liter. The young baby ingesting about 650 cc. of milk receives only about 200 mgm. of calcium daily. Very little calcium is excreted in the urine of such an infant; the minimal fecal excretion, regardless of intake, seems to approximate 150 mgm. Retention of calcium during this early period must be low; the reported values average 48 mgm. daily. As the infant grows and ingests more milk, the amount of calcium ingested and retained increases until the infant of eight months ingests about 350 mgm. and may retain nearly 200 mgm. of calcium a day. The average rate of weight gain of a breast-fed infant is 25 grams daily to 3 months of age, 20 grams daily from 3 to 6 months, and decreases to 15 to 17 grams a day during the last half of the first year. Using these weight gains and data reported in the literature for daily calcium retention of infants fed human milk, the changes in body content of calcium as estimated by Lindberg's method would be as shown in chart 1, curve HM. The percentage of calcium in the body decreases very sharply during the rapid growth of early infancy and then remains constant at a value approximately equal to that of the 6th to 7th fetal month until the infant is 6 months of age or older. During later infancy

with its lower rate of weight increase and larger daily intake of calcium, the percentage calcium content of the body increases slowly, but not until the last quarter of the year is the birth value regained. In prematurely born infants fed human milk only, as is seen from the chart (curve Prem), the reduction in per cent of body calcium is still more drastic, because of the smaller amount present at birth and the low daily intake of calcium. The rate of growth of this group was estimated as 25 grams daily—more rapid growth would result in still greater rate of decrease in relative body calcium content. It is not surprising that prematurely born infants who have been fed only human milk almost invariably develop rickets.

Few data are reported concerning the calcium retention of infants fed human milk and given vitamin D. Such infants seem to show less variability of retention and greater average retention of calcium than infants not given vitamin D. The possible amount of retention, however, is limited by the low intake.

Infants fed diluted cow's milk feedings without added vitamin D show extremely variable, and usually poor retention of calcium. The prevalence of rickets in these infants is evidence that the relative calcium content of the body probably decreases even more rapidly than that of infants fed human milk.

At the other end of the feeding scale is the modern practice of giving undiluted cow's milk treated to give a fine curd in the infant's stomach, and a source of vitamin D. This dietary regimen provides about 3 times as much calcium as does human milk and has the added provision of vitamin D for efficient utilization. The average daily retention of calcium by infants fed cow's milk and 350 to 400 U.S.P. units of vitamin D daily, varies from 100 mgm. for infants under one month to 300 to 400 mgm. for infants from 4 to 10 months of age. During a large part of infancy, the daily calcium retention of these infants is thus as great or greater than the daily intake of the breast-fed infant. The effect of this increased retention on body composition of calcium is shown in chart 1 as the curve *CM*. The rate of weight gain was determined from the actual weights of the infants of the reported studies. It will be observed that even with the marked increase in calcium intake, the infant under 3 months of age still shows a rate of increase in the body content of calcium less than the increase in weight. After 3 months of age the rate of increase in percentage of body calcium approximately parallels that during fetal life and produces a curve similar to that predicted by Moulton for rate of increase in ash content of the fat-free body.

Studies of relation between calcium intake and retention indicate that a direct relation exists between the two at all levels of milk intake studied, and offer no evidence that the maximum possible retention has been attained at any age during infancy. Studies of infants fed calcium salts in addition to milk, or calcium salts as the sole source of this element show that calcium from inorganic sources is well utilized, especially when the food is alkaline in ash and a proper balance between calcium and phosphorus intake is maintained. Short-term studies are reported wherein the total calcium intake is much greater than the amount supplied by cow's milk. These studies indicate also that retention tends to parallel intake. No studies have been reported wherein infants have been given these larger quantities of calcium over sufficient period of time to have attained a stable daily retention. The data are sufficient to show, however, that with the aid of vitamin D, the capacity of the infant to absorb and retain calcium is far greater than that permitted by the amount of calcium provided in human milk.

Other factors than calcium and vitamin D intake affect the amount of retention of calcium. Illness, whether febrile or not, decreases the calcium retention of both breast and artificially fed infants. An increased intake of roughage decreases the retention to some degree, though it seems probable that such decreases are usually transitory. Acid-ash diets are not usually a factor in infancy; however, the ingestion of fruits or vegetables containing oxalates or other organic acid ions whose calcium salts are insoluble will decrease the calcium retention of the infant measurably. The addition of lactose to the diet is reported to increase the calcium retention of young animals ingesting a diet low in calcium. Each infant given milk is ingesting some lactose; whether an additional amount would cause a further increase in the retention of calcium by the infant, remains to be determined.

The mode of excretion of calcium is affected by the diet. Babies fed cow's milk or calcium salts of equal alkalinity generally excrete larger amounts of calcium in the urine than do infants given only human milk. The amount excreted for a given intake varies considerably in individual infants and seems to bear little relation to the amount retained. The maximum daily urine calcium reported for an infant is 286 mgm. daily, excreted by an infant fed a large excess of calcium carbonate; usually the 24 hour excretion is under 20 mgm. Calcium excretion through the skin is negligible.

The serum calcium of the infant at birth is of higher concentration than that of the mother. It may vary from 10 to 13 mgm. per 100 cc.,

but if the mother's diet has been adequate in calcium and vitamin D, is usually above 11 mgm. per 100 cc. For both breast-fed and artificially-fed infants given sufficient vitamin D the serum calcium tends to remain constant throughout the period of infancy at a value of about 1 mgm. below the average level for newly born infants. The normal range for infancy is usually given as from 10 to 12 mgm. per 100 cc.; infants receiving ample vitamin D tend to have serum calcium levels nearer the upper limit of normal, but when the vitamin D intake is barely adequate, the serum calcium approaches 10 mgm. per 100 cc.

The marked difference between the calcium retention of infants given high calcium feedings plus vitamin D and those fed human milk has aroused much discussion as to the question of "supermineralization" of the infant fed cow's milk. Attempts to study the question by sudden dietary changes from the low to the high calcium feeding, and reverse, lead to unsound conclusions, because of the relatively long period of time required to stabilize an infant to a different type of feeding. Rominger's experiment wherein the calcium retention of an infant was studied for 56 consecutive days without evidence of any loss from the body demonstrated conclusively that the infant is able to maintain a consistently high retention of calcium for a long period without intervening periods of loss. The fact that, even with the high retention, the young infant does not quite maintain the proportion of calcium present in the body at birth may be construed as evidence against any excessive amount of deposition.

The curves for percentage of body calcium in infants given human and cow's milk are so dissimilar that either one must assume marked differences in relative proportion of skeleton to body weight (or in amount of fat deposits) or a very wide range of "normal" calcification of bone. The changes in percentage of calcium would necessitate an average decrease in the breast-fed infant of at least 5 per cent in the relative proportion of body weight due to skeleton if the calcium content were even maintained constant. Anatomical data, however, show that the wet weight of the skeleton remains practically a constant proportion of body weight during infancy. For the differences to be attributable to differences in fat content, nearly 70 per cent of the weight gain of the breast-fed infant would be due to fat. A true decrease in calcium content of bone must be assumed. Eliot and Park state "the long bones of the extremities of the healthy fetus at term are almost completely filled with cancellous bone. The marrow cavity does not become well developed until some time after birth. The meaning of this must be that under normal conditions the fetus is born with a store of calcium in the

skeleton." Hamilton has construed this observation to mean that the relative depletion of body calcium in early infancy is normal and is a utilization of excess stores. Against this conclusion is the occasional occurrence of clinical rickets of moderate or greater severity among breast-fed infants and the common occurrence of physiological osteoporosis. The maintenance of a calcium reserve also may prevent severe depletion of the skeleton during illness.

The well-known fact that rickets develops in the growing infant demonstrates the fact that skeletal growth is not wholly dependent on repletion of calcium stores. A comparison of the rates of linear growth of infants whose skeletons contain larger amounts of mineral shows that, while depletion of body stores may not stop growth, the rate of growth increases with the degree of repletion. The rate of growth of infants given 350 to 800 U.S.P. units of vitamin D daily is greater than that of infants fed not over 135 units daily; the babies of the latter group grow faster than infants given no added vitamin D. The daily calcium retention of the 3 groups is in the order of their rates of growth. Both the rate of linear growth and the retention of calcium decrease when the infant is given 2000 units a day or more of vitamin D.

Notwithstanding the increased skeletal content of calcium in infants fed cow's milk and 350 to 800 U.S.P. units of vitamin D, it has been reported that such infants often show deviations from the normal bone pattern at the epiphysial junction. These deviations appear to be similar to those observed in infants not given vitamin D except that in the latter group, such irregularities seem to precede clinical rickets, whereas in the group retaining more calcium, the irregularities are transitory, appearing during the period of most rapid growth and disappearing without change in dietary regimen. This fact has led to much confusion in the literature concerning rickets. Much further study is needed correlating mineral retention and rate of skeletal growth with studies of relative size and density of bone, as well as bone patterns as shown by roentgen ray.

Phosphorus. Body phosphorus is distributed between the skeleton and the nitrogenous tissues. The amount in the skeleton varies with the skeletal content of calcium, the ratio of calcium to phosphorus being reported as 2.21 to 2.23:1. The ratio of nitrogen to phosphorus varies with the different tissues, being about 15.3:1 for muscle and 13:1 for liver, due to the increased lipoid and nucleoprotein content of the parenchymatous tissues. A ratio of 17:1 is often used for the nitrogenous tissues of the body as a whole.

The relative changes in body phosphorus, nitrogen and calcium are

thus interdependent. The percentage changes in calcium have been discussed. The percentage of nitrogen in the fetus increases as steadily as that of calcium throughout gestation (chart 2), therefore the relative phosphorus content must increase likewise. From analyses of full-term fetuses, at birth the body of a 3000 gram infant contains about 60 grams of nitrogen, 25 grams of calcium and 14.5 grams of phosphorus. If the ratio of calcium to phosphorus in bone is 2.23, 11.2 grams of phosphorus would be contained in the skeleton and only 3.2 grams in the soft tissues, making the ratio of nitrogen to phosphorus 18.8 for the

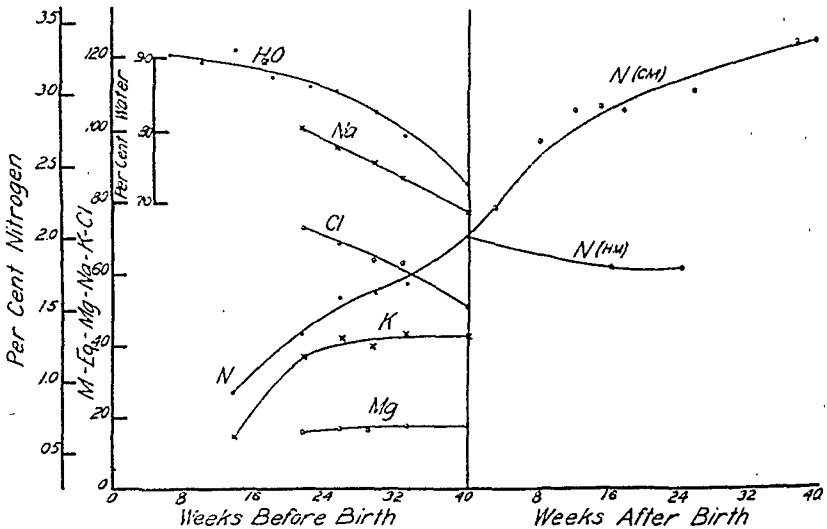


Chart 2. Changes in nitrogen, water and electrolyte content of the fetus, and in nitrogen content of the infant. *CM* and *HM*—infants fed cow's and human milk respectively.

newborn human infant. The fetus at 24 weeks contains relatively more phosphorus in proportion to the nitrogen, the ratio of N:P of soft tissue being 16:1.

The rate of increase in nitrogenous tissues after birth will depend largely on the protein intake. Here again the extremes in customary feeding practices are the low protein feeding of human milk and the high protein intake permitted by the feeding of undiluted cow's milk. The per cent of nitrogen in the body of a breast-fed infant remains at or decreases slightly from the birth value of 2.01 per cent; that of the infant given cow's milk tends to parallel the curve predicted by Moulton for the fat-free body. Changes in both nitrogen and calcium composition

of the body are thus very similar for the same dietary regimens; changes in phosphorus content will follow changes in the other two elements.

The low phosphorus intake of the infant fed human milk results in the retention of a large percentage of the material absorbed. Little if any phosphorus is excreted in the urine. On the other hand, the amount of phosphorus in cow's milk is not only much greater in absolute amount but is greater also in proportion to the calcium and nitrogen content. The proportion of amount absorbed to amount retainable is greater than for human milk. As a result, the kidney becomes the chief organ for excretion of phosphorus, 60 to 70 per cent of the total excretion being in the urine. No evidence of damage to the kidney by this increased phosphorus excretion has been presented.

With phosphorus and nitrogen, as with calcium, the quantity retained by the infant is directly proportional to the intake, if the food is in a form well utilized by the infant. No evidence of maximal absorption or retention has been reported. Thus, the amounts of protein, calcium and phosphorus which can be ingested by an infant fed human milk are far lower than the quantities which that infant is able to digest, absorb and retain. Discussion of compensatory factors is outside the scope of this article.

Blood phosphorus values differ materially in infancy from the values observed in later childhood. At birth, the serum inorganic phosphorus, like the serum calcium, is greater than that of the maternal serum; average values reported vary from 3.5 to 5.4 mgm. per 100 cc., the larger values being observed in blood from infants whose mothers had ingested good diets during pregnancy. The serum phosphorus increases during early infancy, and if the intake of vitamin D is ample, by 6 months of age the maximal values are attained, from 5 to 7 mgm. per 100 cc. The range of serum values is the same for breast-fed and artificially fed infants. By one year of age, serum inorganic phosphorus varies between 5 and 6 mgm. per 100 cc. When the milk intake is ample, but the vitamin D intake inadequate, values tend toward the low normal limits. Values for serum phosphorus below 4 mgm. per 100 cc. are considered indicative of active rickets. The organic phosphorus compounds, both of serum and of corpuscles, tend to be lower at birth than during later infancy.

Magnesium. Magnesium, like phosphorus, is utilized both in the skeleton and the soft tissues. The total quantity, however, is small. The magnesium content of the fetus increases to about 200 to 225 mgm. per kilogram by the sixth month of pregnancy and remains at about this

value throughout the remainder of fetal life. Its partition between bone and soft tissue will be discussed in the next section.

Human and cow's milks show the same relative difference in magnesium as in calcium content. Fewer data are available concerning magnesium metabolism during infancy and such data as we have show a wide variation in retention, the values due in part to difficulty of analysis, particularly with the older methods. The amount of magnesium retained from cow's milk feedings is much greater than that from human milk. From 20 to 50 per cent of the magnesium output is excreted in the urine.

Unlike the serum calcium, serum magnesium in infancy appears to be wholly within the adult range of 2 to 3.5 mgm. per 100 cc. No reports were found as to whether the magnesium content of corpuscles is also at the adult level, 5.4 to 7.8 mgm. per 100 cc.

Electrolytes of body fluids. With exception of the minerals associated with special tissues, including the skeleton, the amount of electrolytes in the body will depend on the amount of body water and its distribution between intra- and extra-cellular fluid. The study of composition of body fluids has progressed rapidly in the past few years, particularly through the efforts of the Yale investigators, and the recent findings may be summarized as follows:

Body chloride occurs almost wholly in extra-cellular fluid; the only exception is the chloride of red blood cells, which amounts to 80 m.-eq. per liter of cellular water. About 25 per cent of the total body sodium occurs in an insoluble form in bone and cartilage; the sodium of bone occurs in the proportion of 1 mM of sodium to 30 mM of calcium. The remainder of body sodium is in extra-cellular fluid. Extra-cellular fluid is of constant composition and contains 120 m.-eq. of chloride, 148 m.-eq. of sodium, 5 m.-eq. of potassium, 1 to 2 m.-eq. of magnesium, and 2 to 3 m.-eq. of calcium per liter of water. The cation content of intra-cellular fluid is also constant, each liter of intra-cellular water containing 140 m.-eq. of potassium and 35 m.-eq. of magnesium; the anions—protein, organic phosphate and bicarbonate—vary in amount with the tissue. Muscle tissue has the lowest protein content, containing 300 grams of protein and 100 mM of phosphate per liter of water; liver contains 450 grams protein and 180 mM of phosphate per liter.

Average values for nitrogen, water and electrolyte content of fetuses from values reported in the literature are shown in chart 2. The body content of extra-cellular and intra-cellular water can be readily estimated from the chloride and potassium content. The chloride content

decreases from about 73 m.-eq. per kilogram body weight at the twentieth week of gestation to 50.4 m.-eq. per kilogram at birth. Without correcting for the small amount of chloride in red blood cells, approximately 62.5 per cent of the body weight of the fetus at the twentieth week of gestation is due to extra-cellular water, whereas only 43 per cent of the weight at birth is ascribable to this source.

The potassium content of the fetus increases rapidly to about 38 m.-eq. per kilogram at 24 weeks in utero, then more slowly to the average value of 43 m.-eq. per kilogram at birth. Estimates of extra- and intra-cellular water from chloride and potassium values (uncorrected) give the following values for fetal water. At 24 weeks the average values are, extra-cellular water 58 per cent and intra-cellular 27.5 per cent; at 32 weeks, 51 and 29 per cent and at birth, 43 and 31 per cent respectively for extra- and intra-cellular water. These values compare very favorably with the average total water content as determined by analysis, 85.5, 79.6 and 72.5 per cent respectively for fetuses at the 24th, 32nd, and 40th week of gestation. The proportion of body weight due to extra-cellular water at birth is still more than double the amount found in the adult, which is 20 per cent of the total body weight. The newborn infant has been likened to the edematous adult in this respect. According to Kerpel-Fronius, throughout life the muscles, skin and nervous system account for 61 per cent of the water content of the body; in the newborn infant, 32 per cent is extra-cellular and 29 per cent intra-cellular water; while in the adult 20 per cent is extra- and 41 per cent intra-cellular. Whereas in the adult the intra-cellular water of musculature accounts for nearly half the total body water, at birth only 18 per cent of the water content is so found. The skin of the newborn infant contains 21 per cent of the total body water, that of the adult, 9 per cent. Iob and Swanson report that fetal bone is 43 to 46 per cent and cartilage 80 to 85 per cent water. Adult bone contains 10 to 25 per cent water. These variations in water content and distribution cause differences in relative amounts of postassium and magnesium, sodium and chloride present in the body of the newborn infant compared with the adult.

The relative nitrogen content of the body increases more rapidly during fetal life than the rate of increase in intra-cellular fluid. At the 24th week of gestation, the body contains about 1.5 per cent nitrogen, which with an intra-cellular water content equalling 27.5 per cent of body weight, would allow 54 to 55 grams of nitrogen, or about 340 grams of protein per liter of intra-cellular water. By the 32nd week of

intra-uterine life, the body nitrogen has increased to about 1.7 per cent and the intra-cellular water to 29 per cent of body weight, permitting 59 grams of nitrogen (365 grams of protein) per liter of intra-cellular water. At birth the average nitrogen content of 2.01 per cent results in a protein content of 405 grams per liter of intra-cellular water—a value higher than that observed in adults and approaching that of glandular tissue alone. Such a value is in keeping with the knowledge that glandular tissue forms a far larger proportion of body weight in the newborn infant than in the adult, while muscle tissue, which contains a much lower quantity of protein per liter of intra-cellular water, is only 25 per cent of body weight of the newborn infant but about 45 per cent of body weight of the adult.

The magnesium content of the fetus remains almost constant at 18 m.-eq. per kilogram body weight during the last 16 weeks of gestation and is apportioned between intra-cellular water and bone. At 35 m.-eq. per liter of water, the fullterm fetus would use approximately 11 m.-eq. per kilogram body weight in intra-cellular fluids, leaving 7 m.-eq. or 84 mgm. per kilogram for deposition in bone.

To summarize, the chief changes occurring in the last half of fetal life are a steady decrease in the relative amount of extra-cellular fluid, as shown by decreases in the sodium and chloride content per kilogram body weight. The amount of intra-cellular fluid per kilogram body weight increases, but at a much slower rate than the decrease in extra-cellular water, consequently the total water content per kilogram of the fetus decreases. The rate of increase in nitrogenous tissues, as estimated from body content of nitrogen and of intra-cellular water is such as to indicate more rapid growth of glandular than skeletal muscle tissue.

After birth, the changes in relative proportions of body electrolytes as determined by retention studies, would be an efficient tool in aiding to apportion weight gain of infants among various tissues. Reports of retention of these elements by infants must be entirely re-evaluated, however, in the light of the findings of Rominger and Meyer, and of Iob and Swanson that in infants fed cow's milk, from 10 to 15 per cent of the total excretion of sodium, potassium and chloride occurs through the skin. More recently, Levine and his collaborators have observed that the amount of excretion through the skin varies with the temperature and humidity of the surrounding atmosphere. These facts preclude the use of correction factors and make possible only the most general of conclusions concerning comparative retention of infants given different types of feeding.

A comparison of apparent retention (uncorrected for loss through the skin) of sodium and potassium by infants fed human and cow's milk shows that the infants fed cow's milk apparently retain twice as much potassium and about one and one-half times to two times as much sodium as infants given human milk. These differences are so great that even allowing for a high percentage of error, it seems safe to assume that the potassium retention of the infant fed cow's milk is considerably above that of the infant fed human milk. Such an assumption is also consistent with the observed nitrogen retention of the two groups (chart 2). Infants fed human milk do not quite maintain their birth percentage of nitrogen; those fed undiluted cow's milk show a continuous increase in relative body nitrogen at a rate comparable to that predicted according to the constant differential growth ratio. Since the body of the infant fed cow's milk contains a greater percentage of protein than that of the breast-fed infant, the amount of intra-cellular fluid must also be greater and a correspondingly larger retention of potassium, magnesium and phosphorus would be expected.

From the wide difference in uncorrected values for comparative sodium retention, it seems probable that the relative content of extracellular water is also greater in the cow's milk fed infant, though the differences in amount may not be as great as the differences in intracellular water. Human and cow milks are calorically approximately equal, though it is customary to give infants cow's milk feedings of somewhat higher caloric value than would be ingested as human milk. The relative weight gains of the infants fed cow's milk are somewhat greater, a larger percentage of weight gain is due to protein and to water, so presumably a smaller amount is due to fat than in the breast-fed infant. From the constancy with which the curves for nitrogen and calcium follow those estimated by Moulton for body composition on a fat-free basis it appears probable that the fat content of infants fed cow's milk is maintained at approximately the birth level, whereas that of infants fed human milk increases markedly, as does the fat content of suckling young of other mammals.

One complete and well-controlled study of retention of organic foodstuffs, water and minerals is at hand. Gordon, Levine and associates have made use of the fact that the sweat glands do not begin to function until very shortly before birth. Therefore, by using premature infants as subjects, errors due to excretion through the skin were more easily avoided. The infants were kept in an atmosphere of constant humidity and temperature and were closely watched for signs of perspiration.

Concurrent determinations were made of retention of water, organic foodstuffs and electrolytes by four infants. Unfortunately for the purpose of this review no infant was given human milk exclusively so the data provide no evidence for direct comparison of the two types of feeding heretofore considered. The nitrogen and caloric intakes, however, varied from infant to infant. In general, the total water retention averaged about 70 per cent of the total weight gain. The amounts of potassium, and phosphorus retained by the group approximated the amounts estimated from the nitrogen and calcium retention and were consistent with tissue gain of the approximate composition of 54 grams of nitrogen per kilogram of intracellular water. The sodium retention approximated that estimated from the retention of chloride $\left(\text{mM Na} = \frac{148}{120}\ \text{mM Cl}\right)$ and calcium $\left(\text{mM Na} = \frac{\text{mM Ca}}{30}\right)$. Body content of both intra- and extra-cellular water seemed to increase with increased retention of nitrogen. The quantity of fat retained by these infants bore no obvious relation either to caloric or protein intake.

Study of changes in quantity and distribution of body water show that a comparatively wide range both of relative proportion of various tissues of the body and also of relative composition of each must be included as normal.

The electrolyte composition of serum, as of other body fluids, tends to remain constant. The sodium, potassium and chloride content of serum water is the same in infancy as in adult life. Serum at birth has less protein than in later infancy, but the differences are not sufficiently great to make the sodium and potassium content of serum appreciably different from those of other periods of life. The potassium and chloride content of water of erythrocytes is also comparable with that observed in adults. The exact amounts of each present in whole blood will depend on the relative amount of corpuscles present, which is an extremely variable factor in early infancy. Whole blood determinations, therefore, have still less meaning for infants than adults.

Sulfur. The sulfur metabolism of infants has been studied by Blaszó. The sulfur content of human and cow's milk is identical, 0.48 per cent, though the protein sulfur content of cow's milk is larger than that of human milk. The infants retained 82 to 98 per cent of the intake, or 0.053 to 0.076 gram per kilogram daily, whereas the sulfur retention of children 3 to 14 years of age varied from 9 to 59 per cent of the intake, or 0.002 to 0.021 gram per kilogram daily. The amount of

sulfur retained per gram of nitrogen retention is also greater for infants than for older children.

Though very little of the ingested sulfur is excreted, the chief avenue of excretion is the urine. Urinary sulfur of infants is characterized by a higher proportion of neutral sulfur and less inorganic sulfate than the urine of older children and adults.

Iron. The iron content of the fetus under 100 grams' weight is approximately constant at about 0.0050 per cent of body weight. During the rapid growth of late gestation the amount increases to 0.007 to 0.009 per cent. Calculated on dry, fat-free basis, however, the iron content per kilogram tends to be constant throughout fetal life.

At birth, the greatest part of body iron is concentrated in the blood. Though the number of erythrocytes is approximately the same as in adults, the concentration of hemoglobin is usually greater than at any period during later life. The exact amount of hemoglobin varies widely among individual infants, from 15 to 28 grams per 100 cc., but seems to average approximately 22 grams per 100 cc. This high concentration is attributed to the low oxygen tension of the placental circulation. It is stated that a considerable proportion of the hemoglobin is in a form somewhat different from the hemoglobin of adults. After the lungs begin to function, less hemoglobin is needed; destruction of the excess begins almost immediately and is completed by the ninth to twelfth week of life, when the hemoglobin reaches its minimum value of 10 to 12.5 grams per 100 cc. During the decrease in hemoglobin, the amount of nonhemoglobin iron in blood corpuscles increases to about 5 per cent of the total blood iron. After the minimum hemoglobin level is reached, the value increases slowly, attaining 11.5 to 13.5 grams per 100 cc. at about five months of age after which the level remains constant or gradually declines throughout the remainder of the first year. Illness, either gastro-intestinal or infectious, causes a decrease in hemoglobin level. The average values obtained at any age seem to vary with the locality, general standard of health and type of infant dietary, as well as the technique of the investigation. The various factors involved are discussed at length by Faxén.

In addition to the normal high iron content of blood at birth some iron is stored in the liver of the newborn infant. Various studies place the amount of liver iron at from 0 to 100 mgm. with an average value of approximately 50 mgm. The smallest values for liver storage were observed in infants born of anemic mothers. The liver content of iron increases for several weeks after birth. Iron is also stored in the spleen

but the small size of this organ makes the total amount of much less importance for iron metabolism than that in the blood and liver.

Probably the two most important factors in iron metabolism of infants are the high concentration of hemoglobin in the blood at birth, and the fact that the blood constitutes such a large percentage of the total body weight of the newborn. The blood volume, like the hemoglobin concentration varies widely, but averages about 15 per cent of the total birth weight according to Lucas and Washburn. The proportion of blood to total weight decreases as the infant grows, and is about 10 per cent at a year of age.

The iron content of other tissues increases with the weight of the tissues. Muscle hemoglobin is the largest factor to be considered; throughout infancy muscle remains at a constant proportion of about 25 per cent of the body weight and contains about 5 mgm. of iron per kilogram. The average amount of body iron at birth is such that if no iron were lost from the body, none need be retained by the infant from the food ingested during the first six months of life, in order to maintain a blood hemoglobin of about 12 grams per 100 cc. Between six months and 1 year of age, about 0.7 mgm. would have to be retained daily in order to maintain the given concentration of hemoglobin.

The hemoglobin level in the blood of premature infants is approximately the same as for full-term infants. Because of their smaller size, the total amount of hemoglobin is much less than for the average normal infant. The period of physiologic blood destruction lasts as long as that of full-term infants and the amount of decrease in hemoglobin may be somewhat exaggerated. Even though no iron were lost from the body during the period of blood destruction, the rapid growth of the infant and the lowered amount of iron at birth would result in a shortage of body iron, and the development of anemia especially after six months of age.

Infants born of anemic mothers often show hemoglobin values within normal limits, though values as low as 13 grams per 100 cc. are reported. These infants seem rarely to have additional stores of iron in the liver and commonly become anemic during later infancy.

Studies of infants under three months of age show that both breast and artificially fed infants tend to lose small amounts of iron during the period of the hemoglobin breakdown, a fact neither unexpected nor alarming. The non-hemoglobin iron of blood at this age is greater than normal, which according to McCance and Widdowson leads to excretion of iron unless the intake is very high. However, the iron intake of the

average baby is meager. The iron content of milk is very low and, especially for human milk, very variable, values from 0.45 to 1.5 mgm. per liter being recorded. According to Wallgren, the higher values are found during early lactation. The iron content of cow's milk as secreted is given as from 0.15 to 0.6 mgm. per liter. Market milk, however, may have an increased content, depending on the length of time the milk is in contact with iron containers.

Feeding additional iron during early infancy apparently does not result in retention unless the amounts given are large; even then retention is only temporary. Opinions as to the effect of iron feeding on the rate and duration of blood destruction vary; evidence has been presented that prophylactic feeding of small amounts of iron shortens the period of physiological hemoglobin breakdown in premature infants and permits a level equal to that maintained by healthy full-term infants.

After three months of age, iron retention is variable, but in general it may be stated that infants fed human milk usually retain very small amounts of iron. Retentions recorded by Wallgren vary from -0.14 to $+0.5$ mgm. daily, with an average retention for four infants of 0.11 mgm. a day. Infants above three months of age fed cow's milk may gain or lose iron, but the amounts involved are negligible whereas a retention of at least 0.7 mgm. is needed daily to maintain hemoglobin level after six months of age. Thus, when the infant is fed only the milk formula, the body is poorer in iron by six months of age than at birth, and becomes progressively poorer with continued growth. The development of nutritional anemia during the last six months of infancy is almost inevitable, therefore, unless iron is given in addition to that supplied from the milk formula. If in addition to the low intake, infection or illness causes loss of iron from the body, the development of anemia is hastened.

It has become customary to feed supplementary foods much earlier in infancy than was formerly the case. Of these foods the two most often used before five months of age are egg yolk and cereal. An egg yolk adds 0.5 to 1.0 mgm. to the day's intake of iron; the iron of cereals varies from 0.2 mgm. per ounce for highly refined cereals to 17.4 mgm. per ounce for those fortified with iron. Vegetables and fruit purées contribute from 0.1 to 0.5 mgm. per ounce. Infants seem to utilize the iron of meat, which is largely organic, as well as inorganic forms of iron; the iron of oxalate-containing leafy vegetables, however, seems not to be utilized by infants. Soluble iron salts, both ferrous and ferric, are well utilized by infants. Iron excretion is almost wholly by way of the

feces. From retention studies, the iron requirement of artificially fed infants over three months old has been placed at greater than 0.5 mgm. per kilogram and close to 1 mgm. per kilogram daily. On the basis of blood studies only, an intake of 25 mgm. of iron and 1 mgm. of copper daily is stated to be necessary for the maintenance of optimal hemoglobin levels. It is difficult to maintain an intake of 1 mgm. per kilogram from food alone; it would be impossible for infants to attain an intake of 25 mgm. of iron daily without the use of iron salts. The actual iron requirement in infancy varies with several factors, not all of which are as yet understood. The low gastric acidity of infants is probably not an important factor except that the anacidity occurring during infection probably contributes to the impaired utilization of iron at that time. The thiamin intake may be a factor. Adequate intakes of vitamin C and of copper are known to be essential for utilization of the retained iron, vitamin C for proper functioning of bone marrow and copper for the formation of hemoglobin.

Copper. The copper content of fetal blood at 20 to 25 weeks' gestation is reported as 0.195 to 0.235 mgm. per 100 cc.; that of full-term infants from 0.073 to 0.082 mgm. per 100 cc. Average values reported for older infants vary from 0.100 to 0.176 mgm. per 100 cc. Lesné and Briskas report that blood levels of iron and copper are reciprocal, and the blood copper of anemic infants is above normal. Copper is stored in the liver at birth, and the amount decreases during infancy.

The copper content of human milk is reported as from 0.21 to 0.28 mgm. per liter; that of cow's milk from 0.09 to 0.21 mgm. per liter. Pasteurization or evaporation in copper tanks increases the copper content of milk. The supplementary foods used in infancy also contain small amounts of copper. The copper requirement of the infant is not known, but it has been definitely established that hemoglobin regeneration is more rapid when anemic infants are given copper as well as iron in amounts greater than those provided by the diet. The ratio of 1 mgm. of copper to 25 mgm. of iron is stated to be most effective in increasing the hemoglobin content of blood. If the iron requirement is placed at 1 mgm. per kilogram body weight, the copper intake should be met by an intake of 40 micrograms per kilogram. Such an intake cannot be met from cow's milk alone.

Cobalt. Cobalt sulfate in doses of 25 to 50 mgm. daily together with 0.5 gram of iron a day is reported to cause an average gain in hemoglobin of 0.75 gram per 100 cc. weekly for 3 to 4 weeks in mildly anemic infants after treatment with iron alone has failed to cause hemoglobin increase. No toxic symptoms were noted during the progress of the study. The

amounts of both substances given seem excessive and the copper content of the salts was not determined. In view of the very small amounts of cobalt needed for cure of anemia in animals and the fact that the amounts reported to produce polycythemia in animals are not large, it seems that the use of cobalt salts for anemia of infants should be attended with caution. Recent studies indicate that cobalt is probably not necessary for normal hemoglobin metabolism in human infants fed milk.

Iodine. The knowledge of iodine metabolism during infancy is still far too scanty. Iodine deficiency in the mother results in hypothyroidism in the infant. In regions where goiter is prevalent the use of iodized salt (1:5000) throughout pregnancy is advocated for normal women as an effective means of preventing thyroid deficiency in herself and her infant. In the Swiss Canton of Bern, where iodized salt contains only 5 mgm. of iodine per kilogram but other forms of iodine prophylaxis are common, the average weight of thryoid in the newborn has decreased from 10 to 6.6 grams in the last thirty years. Scammon states that the weight of thyroid in the normal newborn infant is 1.5 to 2.5 grams and larger thyroids are evidence of iodine deficiency. It appears that the higher iodine content of American iodized salt is a better safeguard than the smaller amount in Swiss salt. Crile and Crile advocate a still larger prophylactic dosage, 10 mgm. of iodine weekly for all non-goitrous pregnant women in regions where goiter is prevalent.

The iodine content of the mother's blood increases during the latter half of pregnancy to from 20 to 40 micrograms per 100 cc., about double the level in non-pregnant women. The iodine content of the cord blood is from 10 to 20 micrograms with an average of about 15 micrograms per 100 cc. In later infancy the average blood iodine decreases [illegible] 5 and 10 micrograms per 100 cc.

As would be expected, the thyroid contains the largest percentage of body iodine at birth. The amount of iodine increases both [illegible] and absolutely with the size of the gland. According to [illegible] normal fetuses, the total iodine content of the thyroid is rar[illegible] micrograms, but in enlarged thyroids the iodine content is [illegible] Palmer and associates found the iodine content of the thyr[illegible] born infants averaged 49 micrograms, 20 per cent of which [illegible] thyroxin. The amounts of iodine found by Palmer in the [illegible] newborn infants equalled the amounts observed by Lelkes [illegible] roids of older infants, values of 48 to 168 micrograms bei[illegible] The thyroids examined weighed 1.5 to 3.0 grams.

The iodine content of both human and cow's milk is vari[illegible]

ing on the iodine content of the food. The percentage amount in colostrum is greater than that of milk, varying from 12 to 45 micrograms per 100 cc. for human colostrum and from 13 to 17 micrograms per 100 cc. for cow's milk. The iodine content of human milk after lactation is established varies from 2 to 15 micrograms per 100 cc.; for cow's milk, iodine values of 0.3 to 187 micrograms per 100 cc. have been reported, the high value being from milk of cattle near the seacoast. The iodine content of cow's milk has been increased about 10-fold by feeding iodine. The iodine of milk is associated with the milk fat. If skimmed milk is fed, or if milk fat is replaced by other fat of lower iodine content the iodine content of the milk feeding will be markedly reduced.

The increased blood iodine at birth and the relatively high iodine content of colostrum are often considered as means of increasing the iodine storage of infants. It is worthy of comment, however, that the actual day's intake of iodine is greater after lactation is established than when colostrum is given, due to the difference in quantity of intake.

Of the other foods in the infant dietary, cod liver oil is one of the best sources of iodine. American cod liver oil contains 3.6 to 15 micrograms of iodine per gram. A teaspoonful daily (3.5 grams) thus adds 12 to 50 micrograms of this element to the day's intake. The iodine content of the other articles of the baby's diet varies even more widely than the iodine content of milk. The amount of iodine in eggs varies according to the iodine intake of the hen. Of foods in general, green vegetables, milk and fruit contain the largest amounts of iodine, cereals much less; the exact amounts present vary with the soil content.

The iodine intake of the young breast-fed infant may vary from about 25 micrograms to as much as 140 micrograms daily, the latter if the milk iodine is high and cod liver oil is given; that of the older infant given cow's milk may be anywhere from 3 to 600 or more micrograms daily.

In view of the variability of the iodine content of human milk and its susceptibility to change with the iodine intake of the mother, the amount of iodine in reported samples of human milk cannot be accepted as a guide to the iodine needs of the infant. The fact that intakes varying from 3 to 600 or more micrograms daily apparently do not result in demonstrable ill effects shows that excess is either quickly excreted or stored, or that with iodine as with many of the other elements considered, the range of body content which must be considered normal is very wide.

In this respect, the findings of Remington and Remington may be of significance, that 50 or 100 times the minimal protective dose for iodine *did not affect growth or utilization of food for growth in growing rats.* Knowledge of optimal iodine requirement in infancy must await further investigation.

Other minerals. Little if anything is known concerning the manganese, zinc or fluorine requirements of infants. The amounts of these substances reported in cow's milk are as follows: manganese, 10 to 40 micrograms, zinc, 3.4 mgm. (average), and fluorine, 0.05 to 0.25 mgm. *to the liter. In districts where the fluoride content of water* is above 0.8 parts per million, infants should be specially protected. Apparently the tolerance for fluorine in food is considerably greater than for fluorine in water.

In conclusion, it may be stated that neither the minimal nor the optimal requirements of the infant are definitely known. Study of the mineral metabolism indicates that a surprisingly wide range of body composition *seems to be compatible with health during the period of* infancy.

The author is indebted to Dr. S. Z. Levine for the privilege of reading his recent article in manuscript form.

REFERENCES

To conserve space, reference is made only to recent and well-documented publications. The reader is referred to these for the older literature.

Introduction

HAMILTON, B. Acta. Paed. 18: 272, 1936.
HUXLEY, J. S. Problems of relative growth. Methuen and Co., Ltd. London, 1932.
LINDBERG, G. Ztschr. f. Kinderheilk. 16: 90, 1917.
MOULTON, C. R. J. Biol. Chem. 57: 79, 1923.
YANNET, H. AND D. C. DARROW. J. Biol. Chem. 123: 295, 1938.

Calcium, phosphorus and magnesium

BOOHER, L. E. AND G. H. HANSMANN. J. Biol. Chem. 94: 195, 1931.
BROCK, J. Ztschr. f. Kinderheilk. 44: 163, 1927.
CZERNY, A. D. AND A. KELLER. Des Kindes Ernährung, Ernährungs-störungen and Ernährungstherapie. Franz Deuticke, Leipzig und Wien, 1928.
DE TONI, G. Arch. Ital. di Ped. e Puericult. 1: 1, 1932; 2: 1, 1933.
ELIOT, M. M. AND E. A. PARK. Brennemann's System of pediatrics. Vol. I, chap. 36. W. F. Prior Co., Inc., Hagerstown, Md., 1938.
GIVENS, M. H. AND I. G. MACY. J. Biol. Chem. 102: 7, 1933.

HAMILTON, B. Boston Med. and Surg. J. **191**: 339, 1924.
Acta Paed. **2**: 1, 1923.
IOB, V. AND W. W. SWANSON. Am. J. Dis. Child. **47**: 302, 1934.
JEANS, P. C., G. STEARNS AND OTHERS. J. Ped. **8**: 1, 1936.
JEANS, P. C. J. A. M. A. **106**: 2066, 2150, 1936.
JEANS, P. C. AND G. STEARNS. J. Ped. **13**: 730, 1938.
JUNDELL, I. AND H. MAGNUSSON. Acta. Paed. **9**: 91, 1929–30.
KRUGER, E. *Ztschr. f. Kinderheilk.* ***53***: *83, 1932.*
LEITCH, I. Nutr. Abs. and Rev. **6**: 553, 1936–37.
MALMBERG, N. Acta Paed. **2**: 209, 1923.
MAXWELL, J. P. Nutr. Abs. and Rev. **4**: 1, 1934.
MUHL, G. Acta. Paed. **2**: Suppl. 1, 1923.
RECTOR, J. M. J. Ped. **6**: 161, 167, 1935.
ROMINGER, E. AND H. MEYER. Arch. f. Kinderheilk. **80**: 195, 1927.
SCAMMON, R. Abt's Pediatrics I. Chap. 3. W. B. Saunders Co., Philadelphia, 1923.
SCHMIDT, C. L. A. AND D. M. GREENBERG. Physiol. Rev. **15**: 297, 1935.
SHOHL, A. T., A. M. WAKEMAN AND E. Y. SHORR. Am. J. Dis. Child. **35**: 576, 1928.
SLYKER, F., B. M. HAMIL, M. W. POOLE, T. B. COOLEY AND I. G. MACY. Proc. Soc. Exper. Biol. and Med. **37**: 499, 1937–38.
SMITH, B. T. Thesis. State Univ. of Iowa, 1938.
SONTAG, L. W. Am. J. Dis. Child. **55**: 1248, 1938.
STEARNS, G. Am. J. Dis. Child. **42**: 749, 1931.
Am. J. Dis. Child. **46**: 7, 1933.
STEARNS, G. AND E. WARWEG. J. Biol. Chem. **102**: 749, 1933.
SWANSON, W. W. Am. J. Dis. Child. **43**: 10, 1932.
TELFER, S. V. Glasgow Med. J. **114**: 265, 1930.
TOVERUD, K. U. AND G. TOVERUD. Acta Paed. **16**: 459, 1934.
TOVERUD, K. U. Acta Paed. **22**: 91, 1937.
White House Conference Reports. Growth and development of the child. III. Nutrition. The Century Co., New York, 1932.
ZAHARESCU-KARAMAN, N., M. ALEXIU AND A. URSU. Compt. rend. Soc. Biol. **122**: 705, 1936.

Electrolytes of body fluids

GORDON, H. H., S. Z. LEVINE, E. MARPLES, H. MCNAMARA AND H. R. BENJAMIN. J. Clin. Invest. **17**: 187, 1939.
HARRISON, H. E., D. C. DARROW AND H. YANNET. J. Biol. Chem. **113**: 515, 1936.
HARRISON, H. E. Ibid. **120**: 457, 1937.
IOB, V. AND W. W. SWANSON. J. Biol. Chem. **122**: 485, 1938.
KERPEL-FRONIUS, E. Ztschr. f. Kinderheilk. **58**: 726, 1937.
ROMINGER, E. AND H. MEYER. Ztschr. f. Kinderheilk. **47**: 721, 1929.
SWANSON, W. W. AND V. IOB. Am. J. Dis. Child. **45**: 1036, 1933.
WHEATLEY, M. A. AND S. Z. LEVINE. Am. J. Dis. Child. **48**: 1432, 1934.
YANNET, H. AND D. C. DARROW. J. Biol. Chem. **123**: 295, 1938.

Sulfur

BLASZÓ, A. J. de Physiol et de Path. gen. **34**: 120, 523, 1936; Acta Paed. **22**: 140, 1937.

Iron, copper and cobalt

Anderson, B. and G. Ortman. Acta Med. Scand. 93: 410, 1937.
Beck, O. München Med. Wchnschr. 79: 378, 1932.
Elvehjem, C. A., W. H. Peterson and D. R. Mendenhall. Am. J. Dis. Child. 46: 105, 1933.
Elvehjem, C. A., D. Duckles and D. R. Mendenhall. Am. J. Dis. Child. 53: 785, 1937.
Fåxen, N. Acta Paed. 19: Suppl. 1: 1937.
Fullerton, H. W. Arch. Dis. Child. 12: 91, 1937.
Gorter, E. Am. J. Dis. Child. 46: 1066, 1933.
Iob, V. and W. W. Swanson. J. Biol. Chem. 124: 263, 1938.
Josephs, H. Bull. Johns Hopkins Hosp. 49: 246, 1931.
Ibid. 55: 259, 1934.
Josephs, H. W. and P. Winocur. Bull. Johns Hopkins Hosp. 61: 75, 1937.
Kato, K. J. Ped. 11: 385, 1937.
Lesné, E., P. Zizine and S. B. Briskas. Compt. rend. Soc. Biol. 121: 1582, 1936.
Lesné, E. and S. B. Briskas. Acta Paed. 22: 123, 1937.
Lichtenstein, A. Acta Paed. 1: 194, 1921.
Lucas, W. P. and A. H. Washburn. Clinical pediatrics. Vol. 11. D. Appleton and Co., New York, 1928.
Magnusson, J. H. Acta Paed. 18 Suppl. 1, 1935.
McCance, R. A. and E. M. Widdowson. Lancet 2: 680, 1937.
Merritt, K. and L. T. Davidson. Am. J. Dis. Child. 46: 990, 1933; 49: 642, 1935.
Nitzescu, I. I. Compt. rend. Soc. de Biol. 106: 1176, 1931.
Oldham, H., F. W. Schlutz and M. Morse. Am. J. Dis. Child. 54: 252, 1937.
Quam, G. N. and A. Hellvig. J. Biol. Chem. 78: 681, 1928.
Sachs, A., W. E. Levine and A. A. Fabian. Arch. Int. Med. 58: 523, 1936.
Schlutz, F. W., H. Oldham and M. Morse. Am. J. Dis. Child. 56: 735, 1938.
Stearns, G. and D. Stinger, J. Nutrition 13: 127, 1937.
Stewart, A. Brit. J. Child. Dis. 34: 1, 1937.
Strauss, M. B. J. Clin. Invest. 12: 345, 1933.
Toverud, K. U. Acta Paed. 17: 136, 1934-35.
Underwood, E. J. and C. A. Elvehjem. J. Biol. Chem. 124: 419, 1938.
Usher, S. J., P. N. MacDermott and E. Lozinski. Am. J. Dis. Child. 49: 642, 1935.
Wallgren, A. Rev. franc. de Pediat. 9: 196, 1933.

Iodine

Crile, G. and G. Crile, Jr. Med. Clin. N. Am. 19: 1715, 1936.
Elmer, A. W. and W. Rychlik. Compt. rend. Soc. de Biol. 117: 530, 1934.
Hanford, A. M., J. C. Supplee and R. E. Remington. J. Dairy Sci. 17: 15, 1934
Holmes, A. D. and R. E. Remington. Am. J. Dis. Child. 49: 94, 1935.
Leipert, T. Biochem. Ztschr. 270: 448, 1934.
Lelkes, Z. Endokrinologie 13: 35, 1933-34.
Maurer, E. and St. Dietz. Biochem. Ztschr. 178: 160, 1926.
Palmer, W. W., J. P. Leland and A. B. Gutman. J. Biol. Chem. 125: 615, 1938.
Remington, R. E. and J. W. Remington. J. Nutrition 15: 539, 1938.

REMINGTON, R. E., J. F. McCLENDON, H. VON KOLNITZ AND F. B. CULP. J. Am. Chem. Soc. **52**: 980, 1930.
STEINMANN, B. Endokrinologie **16**: 395, 1935–36.
TOEPFER, D. Ztschr. f. Kinderheilk. **56**: 405, 1934.

Other minerals

BROEK, A. AND L. K. WOLFF. Acta brev. neerl. Physiol. **5**: 80, 1935; Nutr. Abs. and Rev. **5**: 596, 1935–36.
KAMMERER, A. R. AND W. R. TODD. J. Biol. Chem. **94**: 317, 1931–32.
KEMPF, C. A., D. A. GREENWOOD AND V. E. NELSON. J. Lab. and Clin. Med. **22**: 1133, 1937.
PHILLIPS, P. H., E. B. HART AND G. BOHSTEDT. J. Biol. Chem. **105**: 123, 1934.

PHYSIOLOGICAL REVIEWS

VOL. 19 OCTOBER, 1939 No. 4

THE EFFECT OF TOXAEMIA ON METABOLISM

ERIC HOLMES

The Biochemical Laboratory, Cambridge

The recent progress made in biochemistry, which has depended as well upon the elaboration of new technique, as upon the intensive use of classical methods of investigation, has of recent years made it possible to approach some of the problems of pathology from a new angle. Hitherto, except in the case of certain diseases clearly recognized as essentially "metabolic," such as diabetes, pathological changes have, for the most part, been recognized and described in terms of morbid histology. In conditions in which histological methods can be employed, they have often proved of the utmost value in diagnosis; indeed modern clinical medicine rests upon the foundations of morbid anatomy and morbid histology. Yet anatomical and histological investigation reveals changes in form without necessarily indicating how far such changes may be accompanied by an alteration in function. It may be obvious that the major part of an organ has been destroyed, and replaced by fibrous tissue; and such a finding may be correlated with a clinical picture which is dominated by gross insufficiency in the function of that organ. But such a condition, when far enough advanced to be clearly recognized is often irremediable. On the other hand, material is often available to show that in an acute illness, there are definite changes in cells which can regularly be demonstrated histologically, and which, it is fair to assume, go on, in non-fatal cases, not to destruction and replacement by fibrous tissue, but to repair. The question arises as to what disturbance in function accompanies these lesions. Since the patient is clinically "ill" and yet complete recovery occurs, it is plain that bodily function has been temporarily disturbed. The exactness with which such disturbance can be described, depends, equally obviously, on our knowledge of normal function. In the case of the circulatory system, disturbances can probably be most accurately

described in terms of haemodynamics and electrophysiology. On the other hand, knowledge of the metabolism of organs, such as the liver and the kidney, and perhaps the central nervous system, is increasing so rapidly, that in these cases pathological changes may soon be most readily and most usefully explicable in biochemical terms. Indeed, it seems that the beginnings of such a "chemical pathology" already exist; and, trivial though they are in comparison to the importance which they will doubtless assume in the future, it is the object of this review to gather them together as far as may be possible. Such studies must obviously depend for their inception and inspiration on clinical observation and on the pathological findings in human cases. In many instances they must depend on animal experiment if they are to be carried very far.

It is not intended to review the vast literature which has gathered round the study of diabetes and nephritis. These were recognized from the first as diseases which must be described in chemical terms, and have been investigated by chemical methods. But there is little doubt that the existence of diabetes forced the development of the first satisfactory "micro" method, that for blood sugar, and so has influenced the whole trend of modern biochemistry which depends so largely on accurate "micro" determinations of various substances.

Hibbard and Morrisey (1899) recognized the occurrence of glycosuria in patients suffering from diphtheria, but one of the earliest experimental studies of the effect of toxaemia upon carbohydrate metabolism was made by Rosenthal (1914). He used rabbits, and injected large doses (lethal in 12–48 hours) of diphtheria toxin intravenously. He observed a fall in blood sugar. He noted a fall in temperature, in blood pressure, and a terminal "myasthenia," though he made no mention of convulsions. He discussed at length the previous literature bearing on the morbid histology and adrenalin content of the suprarenal glands in diphtheria, and drew a parallel between the symptoms of suprarenalectomy and those of diphtheritic toxaemia.

He shows that, even if rabbits are given a very high carbohydrate diet before receiving toxin, their livers contain little or no glycogen when they are killed. He argues that, since glycogen disappears without giving rise to a hyperglycaemia, the loss of glycogen cannot be due merely to accelerated glycogenolysis, but must be caused by a failure of the liver to synthesise glycogen. He observes that, after the administration of glucose the hyperglycaemia is more prolonged, and the blood sugar rises more than in normal animals. In late toxaemia,

adrenalin fails to raise the blood sugar. Though the conception of gluconeogenesis was less fully developed than it is at present, yet it is clear that Rosenthal had grasped the idea that in severe toxaemia, the power of the liver to manufacture carbohydrate was impaired. He stressed the probable rôle of suprarenal lesions in the general picture.

Hector (1926) conducted a clinical investigation on diphtheria patients. He observed a "diabetic" type of glucose tolerance curve, with (in severe cases) a low fasting blood sugar value. The urine was acid, the "ammonia coefficient" low, and laevulose tolerance tests indicated some liver damage. The respiratory quotient failed to rise after glucose ingestion. He discusses the probability of endocrine lesions in diphtheritic toxaemia, and notes that two of his cases which proved to be suffering from quinsey, and not from diphtheria, showed no disturbance of glucose tolerance, though they did show an abnormal laevulose tolerance.

Elkeles and Heimann (1927a, b) investigated the blood sugar in cases of human diphtheria. In some of their severe cases they found a low fasting blood sugar, but in others it was normal or high. Some cases gave glucose tolerance tests of a diabetic type, and similar curves were found in cases of diphtheritic paralysis. The ingestion of 40 grams of fructose sometimes gave rise to hyperglycaemia and glycosuria, and they demonstrated insulin resistance in some of their cases, though if the insulin were previously incubated with diphtheria toxin, its power to lower the blood sugar of rabbits was unimpaired. They discussed the possibility that diphtheria intoxication had a "sympatheticotonic" effect, and they showed that ergotamine tartrate largely abolished the rise of blood sugar which followed the ingestion of glucose by their diphtheritic patients.

Mikami (1925) injected very large doses of diphtheria toxin into rabbits. Death followed in most cases within 24 hours. He found an initial hyperglycaemia, followed by a fall in blood sugar, and death in hypoglycaemia. The adrenalin content of the suprarenal medulla fell slightly at first, rose, and fell again. The liver glycogen fell markedly. The decrease both in adrenalin and in liver glycogen was prevented by double splanchnotomy.

Stimulated by often repeated observation of the adverse effects of intercurrent infections upon the course of diabetes, and upon its treatment with insulin, Lawrence and Buckley (1927) investigated the effect of experimental diphtheritic toxaemia upon the response of rabbits to an injection of insulin. The dose of toxin was of a size to

cause death in from seven to eight days in the majority of the animals. It was regularly observed that, during toxaemia, the response to insulin was diminished, and in some cases was altogether abolished. In some animals which survived for seven or eight days, a "pre-lethal" rise of temperature was observed and this was accompanied by a marked hyperglycaemia, though in animals which died in one or two days, a hypoglycaemia was usual. The hyperglycaemia was abolished by injections of ergotamine tartrate, and was therefore ascribed to overactivity of the sympathetico-adrenal system. The authors attribute the resistance to the hypoglycaemic action of insulin to increased glyconeogenesis caused by the stimulation by the toxin of the thyroid and pituitary glands, and they discuss the clinical evidence pointing to a like cause of the disturbances of carbohydrate metabolism met with both in normal and diabetic subjects in infective states. Cramer (1926) had already pointed out that the "thyro-adrenal apparatus" played a large part in the control of body temperature, and had brought together much evidence that lesions of the thyroid and adrenals are regularly found in infective conditions.

Zweckwer and Goodell (1925a) observed, confirming the observation of Menton and Manning (1924), that the injection of heat-killed cultures of certain organisms produced a marked hyperglycaemia in rabbits. The hyperglycaemia produced by similar killed cultures of some other organisms was, by comparison, very slight, while a third group produced no rise in blood sugar at all. The organisms in the first class were B. proteus, B. coli, and B. paratyphosus B; in the second, B. paratyphosus A, and B. enteritidis; and in the third, B. faecalis alkaligenes, Strep. haemotyticus, Strep. viridans, Staph. aureus, B. pyocyaneus, and B. typhosus. Severe symptoms were frequently observed, but were not apparently related to the hyperglycaemia.

Sweeney and Lackey (1928) used rabbits into which they injected a dose of diphtheria toxin which was usually lethal in from five to seven days. Dextrose tolerance tests were carried out by giving by stomach tube 5 grams of glucose dissolved in 25 cc. of water. As the toxaemia progressed, the tolerance of the animals became more and more impaired. After three to four days' toxaemia, the blood sugar two hours after the administration of the glucose sometimes reached 400 to 500 mgm. per 100 cc. of blood, and the curve showed no signs of flattening or dropping at any point. The authors suggest that there is a quantitative relationship between the severity of the toxaemia and the impairment of the glucose tolerance, due to a disturbance either of the

action, or of the production, of insulin. Sweeney (1928) found that the injection of insulin at the beginning of the glucose tolerance test caused a drop in blood sugar, or at least a flattening of the curve. In spite of the insulin, the tolerance was much impaired in toxaemic animals, but it was argued that since the action of exogenous insulin can be demonstrated, the effect must be due to a failure in endogenous insulin production.

Sweeney, Barshop and LoBello (1934), however, on extending these investigations, found that, as toxaemia progressed day by day, more and more insulin had to be given with the standard dose of glucose to keep the glucose tolerance curve within normal limits. The amount of insulin might rise from 2 units on the first day to 12 or 14 on the 5th. Sweeney, Barshop, LoBello and Rosenthal (1934) found that in normal animals the form of the glucose tolerance curve could be kept constant even when the dose of glucose was considerably increased, provided that it was accompanied by a proportionately increased dose of insulin. After diphtheria toxin, however, this was no longer the case; the blood sugar rose much higher as the dose of glucose was increased, despite the extra insulin. These further findings were interpreted as showing that the effect of exogenous, as well as endogenous insulin was diminished in toxaemia.

Schwenkter and Noel (1930), also using rabbits, and employing a similar dose of diphtheria toxin to that used by Sweeney found that *the terminal blood sugar of those rabbits that survived more than 70* hours after the administration of the toxin was higher than before toxin was given, while terminal hypoglycaemia occurred in animals surviving less than 70 hours. The figures for terminal blood sugar, when plotted against survival time, give a remarkably straight line. The animals dying early had terminal convulsions, those dying late were usually in coma for some time before death. A few determinations of liver glycogen gave very low values in all cases. Human patients (children) showed diminished dextrose tolerance and glycosuria after injections of glucose; insulin lowered the blood sugar curve, but the doses used did not bring it back within normal limits.

Schwenkter and Noel (1930b) treated severely toxic cases of diphtheria with insulin and glucose. As large doses of antitoxic serum were also given, the results are of little value.

Evans and Zweckwer (1927) investigated further the hyperglycaemia which occurs after the injection of killed cultures of B. coli and B. Proteus. They found it to be a regular phenomenon; but the rise in

blood sugar could be neutralized by insulin, and the phenomenon was abolished by ergotamine tartrate. They found that if one suprarenal was removed and the other denervated some time before the injection, the hyperglycaemic response was replaced by a hypoglycaemia, which was sometimes fatal. On these grounds they concluded that the hyperglycaemia was due to a stimulation of the suprarenals, which caused a mobilization of sugar from the liver. Thyroid feeding tended to increase the hyperglycaemia, thyroidectomy to diminish it.

In these experiments, as in those of Zweckwer and Goodell, the hyperglycaemic agent was presumably some unknown but heat stable constituent present in the bodies of some (but not of all) bacteria. Zweckwer and Goodell (1925b) found that bacterial anaphylaxis induced in rabbits by injection of killed organisms always resulted in a marked hyperglycaemia, which in one case reached a value of more than 1,000 mgm. per 100 cc. of blood. This phenomenon occurred with all the kinds of bacteria tested, and was therefore independent of the hyperglycaemia which occurs when single injections of some kinds, but not of others are given.

Benn, Hughes and Alstead (1932) showed that the response to the intravenous injection of 20 grams of glucose into human diphtheria patients resulted in a glucose tolerance curve progressively more "diabetic" in type as the cases were more severe. The curve returned to normal as the condition improved, but reverted to the diabetic type if cardiac or nervous sequelae developed. Insulin brought the curves back towards the normal type, and treatment of severe cases with glucose and insulin, as well as with anti-toxic serum was tried, with some apparent success when the results were compared with a previous series of cases which had received serum only.

Williams and Dick (1932) looked for glycosuria in a number of human cases of acute infectious disease. In 41 per cent of the cases it was found; it was usually reduced by insulin administration. It was accompanied by diminished dextrose tolerance, and the tolerance was improved by giving insulin. Most patients examined were suffering from scarlet fever, but the series also included examples of diphtheria, pneumonia, influenza, tonsillitis, measles, erysipelas, encephalitis, mumps, epidemic meningitis, and poliomyelitis. They found diminished dextrose tolerance in rabbits after the intravenous injection of 48 hour cultures of the following organisms: Streptococcus of scarlet fever, Pneumococcus type I, Pneumococcus type III, B. typhosus, B. paratyphosus B, B. influenzae, Streptococcus of erysipelas, scarlet fever anaerobic organisms.

Corkill (1932) observed that injections of diphtheria toxin abolished the power, possessed by normal young rabbits, to lay down glycogen in the liver in response to an injection of insulin or of adrenalin. This occurred even after toxaemia of short duration, when no histological lesions were visible in the liver and when the injection of glucose still gave rise to a deposition of liver glycogen. Toxaemia of 24 hours' duration also made the animals resistant to insulin. After a dose which would normally cause convulsions the blood sugar fell to about 60 mgm. per 100 cc., but then rose again spontaneously, and would do so several times in succession in response to repeated doses of insulin. Adrenalin at this stage of toxaemia produced somewhat less hyperglycaemia than in a normal animal. T. R. Elliott (1912) found that, provided that the splanchnic nerves were intact, diphtheritic toxaemia lowered the adrenalin content of the suprarenals. Corkill found that, histologically, the glands showed loss of chromaffine tissue, which progressed with the severity of the toxaemia; while the organs did not show obvious signs of damage, their adrenalin content was diminished. Since the insulin resistance was abolished by ergotoxin, it was interpreted as being due to an increased secretion of adrenalin.

Corkill and Ochoa (1934) found that while lactate, injected intravenously into young rabbits which had been starved for 24 hours, gave rise to a definite deposition of liver glycogen, no such deposition occurred if the animals had been injected 24 hours previously with diphtheria toxin. Since they did not detect any impairment in the power of muscle extract made from such rabbits to form lactic acid from glycogen or from hexose diphosphate, and since glycogen was still deposited in their livers after an injection of *glucose,* they concluded that the power of the liver to form glycogen from lactate was impaired; they thought that this might be due to a direct effect on the liver cells, but did not exclude an effect on the suprarenal cortex.

Cross and Holmes (1937) were able to confirm that, in vivo, little glycogen could be deposited after an injection of lactate into a 24 hour toxaemic rabbit; they further noticed that, although glycogen was still deposited after an injection of glucose, the amount was distinctly less than in a normal animal after a similar injection.

Soskin, Allweiss and Mirsky (1935) investigated the dextrose tolerance curves of normal and depancreatized dogs an hour or so after a massive intravenous injection of diphtheria toxin. Soskin and his collaborators had previously developed the thesis that the form of the normal dextrose tolerance curve depended on the "homoeostatic" power of the liver rather than upon a secretion of insulin evoked from

the pancreas to deal with hyperglycaemia, and they had shown that normal dextrose tolerance curves could be obtained in depancreatized dogs[1] if the test injection of glucose were superimposed upon a steady intravenous infusion of glucose and insulin so adjusted as to keep the blood sugar level constant. (Soskin, Allweiss and Cohen, 1934; Soskin and Allewiss, 1934; see also Soskin, Essex, Herrick and Mann, 1939). They therefore argue that an important factor in the mechanism for blood sugar regulation is the power of the liver cells to regulate their intake or output of sugar in accordance with the blood sugar level at any moment ("homoeostatic" power). After the injection of diphtheria toxin, the normal, and still more the depancreatized dogs showed a grossly diabetic type of sugar tolerance curve, even though in both cases control curves, obtained immediately before the injection of toxin, were of the usual form. They inferred from this that the liver cells were being affected by the toxin, which, they believed, altered the sensitivity of the liver to the control of the endocrine secretions.

In a further paper Soskin and Mirsky (1935) investigated the effects of rather smaller doses of diphtheria toxin (lethal in about 10 hours) administered to dogs. When no glucose was given there was a slow rise, with a terminal sharp decline in blood sugar; the animals died in hypoglycaemia. If glucose tolerance tests were performed, the results depended to some extent upon the size of the dose of glucose administered. The first change was the appearance of a "diabetic" type of curve, caused, the authors believe, by a derangement of the "homoeostatic" mechanism in the direction of increased glycogenolysis. This might be followed by the reappearance of a normal curve. In the later stages, the curve was of an extreme "diabetic" type. Similar curves are seen after hepatectomy, and so probably correspond to a complete failure of liver function, which, had no glucose been given, would have resulted in a severe hypoglycaemia. It is of interest that the authors do not record that the administration of glucose prolonged the survival time of the animals.

It will be clear from the work which has been discussed that there

[1] In connection with the theory of the "homoeostatic" mechanism of the liver, it is worth remarking that, while Zunz and La Barre (1927) used dogs, many of the experiments which have given support to the theory that the blood sugar is controlled by nervous influences acting on the pancreas via the vagus nerves have been carried out on the rabbit. Hoet found that sections of the vagi in the dog, without or with removal of the kidneys, failed to modify the glucose tolerance curve as it does in rabbits. It may therefore be that the "homoeostatic" mechanism is more active in the dog than in other animals.

is abundant evidence that in toxaemia, whether occurring in human cases or produced experimentally in animals, there is a disturbance, more or less severe, of the normal metabolism of carbohydrate. The knowledge that diabetes, the classical example of disturbed carbohydrate metabolism, was due to deficiency of an internal secretion of the pancreas, and the discovery of the effects of adrenalin upon the blood sugar level, has naturally disposed workers to seek for an explanation of their results in an effect of the toxin upon one or more of the endocrine glands, the more so since there is a very extensive literature dealing with the histological changes observed in different endocrine organs in toxaemic conditions. (For references see Elliott, 1912; Rosenthal, 1914; Cramer, 1926; Elkeles and Heimann, 1927.) While a discussion of this aspect of the matter is beyond the scope of this review, there is not the smallest doubt but that lesions which can easily be recognized histologically appear in the endocrine glands at an early stage in many toxaemic conditions. It is, however, only very recently that it has been possible to appreciate how complicated is the endocrine control of metabolism. The earlier workers in the field were not able to visualise very much more than a disturbance, in the direction either of increased or decreased secretion, of the adrenal medulla, the islets of Langerhans, or the thyroid; for the functions of the adrenal cortex, the anterior lobe of the pituitary, and the parathyroids were almost unknown. We therefore propose to discuss (as far as may be in the light of recent developments) the evidence that the metabolic changes observed in toxaemia are caused by damage to the endocrine glands, without prejudicing the question whether such damage is, in fact, the sole or most fundamental cause, or whether damage to such organs as the liver does not also play a very important part.

Recent work upon the function of the anterior pituitary and the suprarenal cortex has profoundly modified our conception of diabetes, and it is clear that the effects of toxaemia must be interpreted if possible in the light of this knowledge. All observers agree that, in toxaemia, the glucose tolerance curve is modified in the sense that after a standard dose of glucose, administered either by mouth or intravenously, the blood sugar shows a more pronounced and more prolonged rise than usual—that, in fact, the tolerance curve becomes more "diabetic" in type. Clearly enough, this could result from one or more than one of a number of different causes: a failure in the utilization of glucose, a failure in its storage, or a failure in some mechanism by which gluconeogenesis is normally checked when glucose is introduced into the

blood stream from without (this might correspond to the "homoeostatic" mechanism of Soskin). Any or all of these mechanisms may be controlled by the endocrine glands, and may be affected by damage which falls primarily upon the endocrine system.

Rosenthal (1914) in a penetrating discussion of his results, was one of the first to compare the carbohydrate metabolism of experimental diphtheritic toxaemia with the then recognized effects of suprarenalectomy. He used massive doses of toxin, administered intravenously, and the most striking results were hypoglycaemia, circulatory collapse, and the almost complete absence of liver glycogen. (He also concluded that, since liver glycogen disappearance was associated with hypo- and not with hyperglycaemia, it is probable that carbohydrate formation was diminished.) Hypoglycaemia, low blood pressure, and a liver poor in glycogen are of course features of acute suprarenal deficiency, and so far the parallel holds good. But Rosenthal used overwhelming doses of toxin. With smaller doses, and in the earlier stages of toxaemia, the fasting blood sugar is normal or slightly raised (Schwenkter and Noel, 1930; Lawrence and Buckley, 1927; Soskin and Mirsky, 1935); it is only after massive doses, or in the later stages of toxaemia brought about by more moderate doses, that hypoglycaemia occurs. But abnormal sugar tolerance tests and insulin resistance are regularly observed at all periods, and in early toxaemia the liver glycogen may be low (Corkill and Ochoa, 1934; Cross and Holmes, 1937).

After suprarenalectomy, as in early toxaemia, glycogen can be deposited after glucose administration (Deuel, Hallman, Murray and Samuels, 1937) and both toxaemia (Corkill and Ochoa, 1934; Cross and Holmes, 1939; Dawson and Holmes, 1939) and suprarenalectomy (Britton and Silvette, 1934; Buell, Anderson and Strauss, 1936) interfere with the deposition of glycogen after the administration of lactate.

A constant finding of those who have worked on toxaemia has been that both human patients and animals rendered toxaemic experimentally are resistant to insulin. There is, on the other hand, no reason to believe that suprarenalectomy increases insulin resistance—rather the reverse, for subsequent suprarenalectomy diminishes the glycosuria and hyperglycaemia which follow pancreatectomy (Barnes, Scott, Ferrill and Rogoff, 1933).

Bamberger, Neuer and Oelkers (1938) showed once again decreased tolerance to glucose and galactose in diphtheria patients. They also published some not very satisfactory figures for the glycogen content of heart, liver, and skeletal muscle in diphtheritic toxaemia in animals.

Like Harding (1921) they stress the analogy between this condition, Addison's disease, and suprarenalectomy.[2]

Thaddea (1935) published observations on a case of Addison's disease. He was struck by the fact that infections greatly exacerbated the condition, precipitating crises which could be surmounted by the intramuscular administration of cortical extract. During these crises there was hypoglycaemia. The blood sugar was restored to normal by the administration of sufficient extract.[3]

He discusses the relationship between toxic conditions and suprarenal cortical insufficiency. *He claims to have greatly prolonged the lives* of guinea pigs injected with diphtheria toxin by treating them simultaneously with cortical extract and vitamin C. He has discussed this view further (1936a, b). (The alleged effect of vitamin C will be discussed separately.)

The evidence that adrenal cortical extracts exert a protective or curative effect in infective states is fairly strong. Scott and Bradford (1930) showed that adrenalectomized rats were more susceptible than normals to injections of killed B. typhosus cultures. The thymus hypertrophied in these animals after adrenalectomy.

Perla and Marmorsten-Gottesman (1930a) showed that the resistance of adrenalectomized rats to injections of typhoid vaccine and to histamine (1931b) was increased by cortical extract. Hartmann and Scott (1930) report similar results so far as typhoid vaccine is concerned.

Zwemer and Jungeblut (1935) found that guinea pigs which had received cortical extract showed cutaneous lesions of less severity than the controls after the intracutaneous injection of 1/500 to 1/50 M.L.D. of diphtheria toxin. If the extract was mixed with a lethal dose of toxin, and the mixture injected, a greater proportion survived of the animals so treated than of the control group receiving toxin alone. When extract and toxin were injected separately, a less marked protec-

[2] They also find a marked increase both in the oxygen consumption and the anaerobic glycolysis of the suprarenal cortex of diphtheritic animals as compared to that of normals. The measurements were made by shaking slices of the organ in a Warburg apparatus.

[3] Thaddea investigated other aspects of this patient's metabolism, finding a drop in total blood cholesterol, almost entirely accounted for by a decrease in the ester fraction, a rise in blood non-protein nitrogen and blood calcium and a fall in serum chloride. There was a low output of urine and of NaCl, and a high output of creatin. There were signs of renal damage (albumen and casts in the urine). The blood pressure was, of course, abnormally low, and the rise after adrenalin injection unusually small.

tive effect was observed. Administration of massive doses of cortical extract ("Eucortone") to rabbits injected with diphtheria toxin only partly restores their power to deposit liver glycogen after the injection of lactate (Dawson and Holmes, 1939).

There is reason to believe the suprarenal damage or destruction exerts a definite effect on striated muscle. Muscular weakness is a prominent feature of Addison's disease. Ingle (1936) claims that adrenalectomy in the rat diminishes the amount of muscular work which the animal is able to perform. Buell, Strauss and Andrus (1932) claim that autolysing cat's skeletal muscle forms less lactic acid than normal if the animals have been previously adrenalectomized and are suffering severe adrenal deficiency. But Corkill and Ochoa believed that toxaemia did not affect the power of muscle extract to produce lactic acid from glycogen or hexose diphosphate. Diphtheria patients do not appear to suffer from more marked muscular weakness than do those who are as gravely ill from other diseases.

It is well known that the suprarenal cortex is very rich in vitamin C. The organ undergoes change in scurvy, and a marked decrease in its vitamin C content has been recorded in various infective conditions, including experimental diphtheritic toxaemia (Harde and Benjamin, 1935; Lyman and King, 1936; Harris, Passmore and Pagel, 1937). From time to time claims have been advanced that it exerts a protective or curative effect in toxaemia, particularly in experimental diphtheritic toxaemia (e.g., Thaddea, above). Unfortunately, some of this work appears to have been carried out in a most uncritical manner, and the results carry very little conviction. Thaddea's (1935) claims have already been mentioned. Lotze and Thaddea (1937) state that vitamin C, glutathione, and cysteine will prolong the survival time of guinea pigs into which diphtheria toxin has been injected, and that it will also postpone the appearance of histologically demonstrable lesions in the livers, spleens, and suprarenals of such animals. Their published results are very unconvincing. Jungeblut and Zwemer (1935) claim that exposure of diphtheria toxin to ascorbic acid (vitamin C) in vitro destroys its toxicity (but see Sigal and King, below). Injections of ascorbic acid into guinea pigs simultaneously with diphtheria toxin results in the survival of about half the animals, the dose of toxin used normally causing 100 per cent mortality. Previous injection with ascorbic acid diminishes the severity of local reaction which follows the intracutaneous injection of sub-lethal doses of toxin. Jeney, Gagyi and Baranyai (1936) claim that guinea pigs poisoned with diphtheria

toxin live longer on a vitamin C rich diet than on a scorbutic one. King and Menten (1935) found that guinea pigs maintained at a low level of vitamin C intake were more susceptible to sub-lethal doses of diphtheria toxin than were animals receiving normal or large amounts of vitamin. Such animals, too, showed more severe local reactions. Hanzlik and Terada (1936) claim that vitamin C partially protects pigeons from diphtheria toxin and Sigal and King (1937a) claim that the glucose tolerance curves of guinea pigs on a low vitamin C intake which have been injected with diphtheria toxin are more abnormal than those of guinea pigs receiving toxin but on a higher vitamin C intake. None of these workers record anything like 100 per cent protection by vitamin C against 1 M.L.D. of toxin, no matter how much vitamin was administered. One gains the impression that the effect is certainly non-specific, and is probably not very important.

Torrance (1937) has shown that while vitamin C reacts reversibly with diphtheria toxin in vitro, there is no relationship between the potency of the toxin and its effect on the vitamin; that heated and therefore inactivated toxin still reacts with the vitamin, and finally that toxin which has reacted with the vitamin retains its potency. Sigal and King (1937b) show that vitamin C has no action on diphtheria toxin in vitro if it is neutralized and buffered before being mixed with the toxin. This obviously throws doubt on the experiments mentioned above which were adduced in evidence that vitamin C inactivated toxin "in vitro". Zilva (1937) categorically denies that vitamin C protects guinea pigs against diphtheria toxin. Herbrand (1935), Messer (1936) and Bernhardt (1936) support Thaddea's contention (above, p. 449) that the simultaneous administration of vitamin C and suprarenal cortical hormone exert a protective effect in experimental and clinical diphtheritic toxaemia, though neither alone is effective. Since both Messer and Bernhardt gave other forms of treatment, including serum, along with the cortical extract and vitamin C, their evidence is valueless. Dawson and Holmes (1939) find that early (24 hrs.) diphtheritic toxaemia very much retards the rate at which lactate disappears from the blood stream after it has been injected intravenously: if massive doses of suprarenal cortical extract are injected, the rate at which lactate disappears is increased, but is not restored to normal.

It will be seen from the above discussion of the literature that there is a considerable body of opinion which seeks to refer some of the important metabolic changes occurring in toxaemia to lesions of the suprarenal cortex. That such damage may account for some of the phe-

nomena observed is highly probable, but that it is the most important cause of the metabolic disturbances does not seem, to the writer at least, to have been established.

Before the functions of the suprarenal cortex were at all understood, attention was focussed on the suprarenal medulla, and the pioneer workers in this field (e.g., Rosenthal, 1914; Cramer, 1926; Lawrence and Buckley, 1927; Evans and Zweckwer, 1927) were disposed to consider that some or all of the changes in toxaemia were to be attributed to hypersecretion, perhaps later followed by hyposecretion, of the adrenal medulla and possibly of the thyroid. Giganticism and acromegaly are well recognized results of hypersecretion of an endocrine gland which are sometimes caused by infective processes, and apart from this there is a formidable body of experimental evidence pointing to the importance of the suprarenal medulla in toxaemic conditions.

One of the most constant findings has been that toxaemia causes resistance to the hypoglycaemic action of insulin, and a "diabetic" type of glucose tolerance curve. Lawrence and Buckley (1927) and Corkill (1932) found that the insulin resistance of toxaemic rabbits was abolished by ergotamine tartrate. The glucose tolerance curves of some of the patients studied by Elkeles and Heiman (1927) were restored to normal by the same substance. Evans and Zweckwer (1927) found that the hyperglycaemia following injection of killed bacterial cultures into rabbits was abolished by section of the splanchnics, and Mikami (1925) reports similarly on the hyperglycaemia which he observed after the injection of diphtheria toxin. It is now recognized that adrenalin may have a twofold effect upon liver glycogen: on the one hand it causes glycogenolysis, while on the other, by increasing the blood lactate, it subsequently causes an increased deposition of liver glycogen. It is believed (see discussion by Marks (1936)) that the "recovery phase" of the blood sugar which follows a fall produced by insulin is caused by an outpouring of adrenalin, and Barnes, Dix and Rogoff (1933) have shown that the denervation of the suprarenals and destruction of their medullae renders dogs "insulin sensitive."

Cross and Holmes (1937) demonstrated the synthesis of glycogen by slices of rabbit's liver "in vitro." They found that the phenomenon did not occur when the liver slices were taken from a rabbit injected 24 hours previously with diphtheria toxin, and they found also that glycogen synthesis was inhibited when a very small quantity of adrenalin was added to the fluid in which slices of the livers of normal rabbits were suspended. Kotliarov (1938) suggests that extracts of the livers

of cats from which the suprarenal medullae have been removed have less glycogenolytic power than extracts from the livers of normal cats. So far, therefore, as resistance to insulin, absence of liver glycogen, and perhaps the abnormal glucose tolerance are concerned, the facts seem to fit with the supposition that the adrenal medullae are over-active in toxaemia. But again we are confronted with other observations which make it extremely unlikely that such a hypothesis can afford a complete explanation of the facts. If we are to account for the phenomena by assuming an increased secretion of adrenalin we must expect hyperglycaemia and a raised blood pressure regularly to occur. A very great many observations have been made by different workers upon the fasting blood sugar level of human patients and animals in diphtheritic and other toxaemias. There is no evidence that, in early toxaemia, the fasting blood sugar level is consistently and markedly raised, though most workers record a few cases in which this was so. In severe toxaemia, on the other hand, hypoglycaemia is very consistently observed, and animals often die in hypoglycaemic convulsions. Only in some of Lawrence and Buckley's and Schwenkter and Noel's rabbits, which died in 5 to 7 days (most workers have used doses of toxin lethal in 72 hours or less) was hyperglycaemia often observed. The first named workers record hyperthermia and intense hyperglycaemia as an occasional terminal phenomenon, and the last named noted that animals which survived for more than 72 hours often died in coma, while those dying after shorter periods usually died in convulsions, presumably hypoglycaemic in nature. By contrast the injection of some kinds of living (Williams and Dick, 1932) and dead (Zweckwer and Goodell, 1925a) organisms regularly gave rise to hyperglycaemia, and anaphylactic shock may be accompanied by extremely high blood sugars (Zweckwer and Goodell, 1925b). Thus, if we are to explain the regularly occurring insulin resistance, abnormal glucose tolerance curves, and low liver glycogen values solely by an increase in the activity of the suprarenal medulla, then we must expect to find a regularly occurring and marked hyperglycaemia—which is conspicuous by its absence. Similarly, we should expect an increased blood pressure, whereas low blood pressure is a most striking feature of experimental diphtheritic toxaemia. While therefore there is strong evidence that over-activity of the suprarenal medulla may account for some of the important metabolic disturbances in toxaemia, it is at present difficult to believe that it can account for the others, probably equally important. Edmunds and Johnston (1928) and Myers (1933) have observed that

in the late stages of experimental diphtheritic toxaemia adrenalin ceases to have its accustomed effects on the organs innervated by the sympathetic system. Myers found that the adrenal glands of the toxaemic animals still contained appreciable quantities of adrenalin[4], for extracts made from them would produce the usual effects upon isolated or perfused organs, and other preparations from normal animals. In spite of this, the heart, intestines, etc. of the toxaemic animals quite failed to respond either to extracts of fresh adrenals, or to commercial preparations of adrenalin. Pituitrin and nicotine also failed to bring about their usual effects on the heart and intestine, and digitalis added to the isolated perfused toxaemic heart produced its usual "toxic" effect, which was not, however, preceded by a phase in which the amplitude of the beat was increased as it is in the normal heart. These findings suggest that diphtheria toxin exerts a profound effect upon the tissues, as well as upon the endocrine glands.

Recent work on the anterior pituitary has emphasized so strongly its rôle as a regulator of carbohydrate metabolism that the possibility that it plays its part in the phenomena of toxaemia cannot be ignored.

Cope and Marks (1934) found that the injection into rabbits of crude anterior pituitary extracts produced in these animals insensitivity to the hypoglycaemic action of insulin. Young has published a series of papers showing that this substance, which is not capable of a general "diabetogenic" action, can be separated from a number of other physiologically active fractions of the anterior pituitary. He has named it the "glycotropic factor" (Young, 1938b; other references in the paper cited). The action of this factor perhaps furnishes a counterpart to the insulin sensitivity occurring after hypophysectomy.

Himsworth and Scott (1938), repeating the observations of Mann and Bollman (1935) that insulin accelerates the fall of the blood sugar which occurs in a hepatectomized animal, find that this acceleration is inhibited in animals which, before hepatectomy, had received an injection of the "glycotropic" hormone; although the rate of the fall of the blood sugar which regularly occurs in the absence of injected insulin is in no way affected.

Himsworth has developed the view that diet is an important factor in determining both the sensitivity of normal human beings and animals (rabbits) to insulin, and also the form of the glucose tolerance curve. Diets high in carbohydrate increase insulin sensitivity and glucose

[4] C. A. Ashford (personal communication) has confirmed this.

tolerance ("Straub-Traugott phenomenon"); diets low in carbohydrate cause insulin resistance and lower glucose tolerance (Himsworth, 1934) (for summary of work with normal and diabetic patients see Himsworth, 1932, 1936).

It is thus possible that diet may modify insulin sensitivity and glucose tolerance by an effect upon the anterior pituitary. So far as the writer is aware, there is so far no direct clinical or experimental evidence to connect these phenomena with the insulin resistance and decreased glucose tolerance of toxaemia; yet histological changes in the anterior pituitary have been reported as occurring in such conditions. Both human beings and animals suffering from severe toxaemias are likely to refuse food. Starvation decreases glucose tolerance even in the normal organism. It is well recognized not only that the occurrence of infection markedly increases the insulin requirements of diabetics, but that, apart from infections, certain diabetics belong to an "insulin resistant" group. Such cases have been investigated by Himsworth (1936; and other publications to which references will be found in that cited) who draws a parallel between their case and that of patients rendered "resistant" or "insensitive" to insulin by a carbohydrate poor diet. The fact that a diabetic-like condition (Young, 1938b) can be induced in animals by injections of anterior pituitary extract (a counterpart to the amelioration of the symptoms of pancreatectomy brought about by removal of the pituitary) makes it almost impossible, *apart from the work already mentioned, not to seek for a connection* between insulin "resistance" brought about by diet, and the activity of the anterior pituitary; and also to explore the possible importance of lesions of the anterior pituitary in the alterations of carbohydrate metabolism seen in some, at least, of the phases of toxaemia.

It will be obvious from our discusson of the effects of the endocrine glands that it is in the highest degree probable that toxaemia may act primarily upon them, and thereby modify metabolic processes. Important, however, as the endocrine glands may be as regulators of metabolic processes, the fact that animals deprived of pancreas, and suprarenals, or pancreas and pituitary will survive for some time makes it clear that the bodily functions may be maintained without either stimulation or control by these glands. Moreover, the actual work of metabolism is carried on by tissues such as the liver, kidneys, brain and skeletal muscles. In toxaemia these organs often suffer damage which can readily be detected by histological methods, and biochemical, no less than structural lesions may reasonably be expected. Whether the

anterior pituitary, thyroid, adrenals and pancreas are damaged or not, the liver cannot be expected to store and liberate glycogen, to carry out the work of gluconeogenesis, or to deaminate amino acids and form urea in a normal manner if its enzyme systems are deranged by toxaemia. In cases of severe liver damage hypoglycaemia is known to occur, often as a fatal event, and there seems no need to attribute it to any cause other than a failure of liver function, and in particular of the power of gluconeogenesis.

Yannet and Darrow (1933a, b) have approached the question of diphtheritic toxaemia from this point of view. In their first paper, they report the effect of diphtheritic toxaemia upon the blood sugar, blood non-protein nitrogen, blood amino nitrogen, and blood lactic acid. Their results, so far as blood sugar was concerned, conformed to those of other workers. With large doses of toxin, there was either a small rise, no change or a slight fall at the end of 24 hours, followed by hypoglycaemia after 48 hours. With smaller doses of toxin there was hyperglycaemia. The blood non-protein nitrogen rose in all cases; in a few of the less severe cases, the blood amino nitrogen fell again, but in the remainder it rose. The blood lactic acid showed a steady rise in all cases.

In their second paper, they studied the effect of diphtheritic toxaemia on the power of the liver, cardiac muscle, and skeletal muscle to synthesise glycogen from glucose, with and without insulin. So far as initial values were concerned they observed that while toxaemia very much depleted the glycogen content of the liver, it hardly affected that of skeletal and cardiac muscle. The power of the liver in toxaemia to lay down glycogen in response to injections of glucose was much less than in the normal animal (cf. however, Corkill and Ochoa; Cross and Holmes) and this was little affected by the simultaneous injection of insulin. On the other hand, in toxaemia the heart muscle deposited rather more glycogen in response to injections of glucose, and distinctly more in response to injections of glucose and insulin, than did normal heart muscle. The authors interpret these results as indicating a failure in the glycogenetic power of the liver.

The development of the technique for studying the metabolism of slices of tissue suspended in Ringer's solution or other medium, to which various substrates, drugs, etc., may be added at will, has opened up a field of investigation which promises to be useful in the study of the effects of toxaemia upon individual organs. It is obvious that those who employ such methods must be on guard against errors in interpre-

tation; but so far similar methods employed in work on normal tissues have met with marked success, and again and again have confirmed and extended the conclusions reached in studies on the whole animal by the classical methods of biochemistry. The use of the isolated tissue slice in the study of the metabolism in toxaemia obviously permits metabolic studies to be correlated closely with histological examination. The metabolic behaviour of slices of an organ can be observed, and a portion of the same organ used for microscopical section. That metabolic studies on isolated slices will permit the changes observed to be ascribed to the effects of the toxin directly upon the organ studied rather than indirectly through the endocrine system is probable, but not certain. During the course of the experiment, no hormone can reach the tissue unless it is deliberately added by the experimenter, but clearly, unless the tissue is from an animal from which the endocrine gland under consideration has been removed some time previously, it may have carried with it a supply of the substance sufficient to last during the period of the experiment. This possibility can be explored, but it will require time to do so. In the meantime, it is readily possible to demonstrate that marked differences exist between the metabolism of isolated liver tissue from normal and from toxaemic animals. The power of liver to form carbohydrate from non-carbohydrate substances (gluconeogenesis) has been often demonstrated in the whole animal and in the isolated perfused liver. That it is possessed by isolated slices of liver was shown by Gemmill and Holmes (1935), Bach and Holmes (1937) and Cori and Shine (1936); and that it is damaged in diphtheritic toxaemia was shown by Cross and Holmes (1937). They found that after toxaemia of 48 hours' duration in rabbits which had received a dose of toxin lethal in 56 to 72 hours, the power of the isolated liver slices to form fermentable carbohydrate from lactic acid, pyruvic acid, and alanine was diminished by 70 per cent or more. The power to form fermentable carbohydrate from glycerol, glycerophosphate, glyceric aldehyde, and butyrate, was also diminished, but to a lesser degree. After only 24 hours' toxaemia, the diminution of gluconeogenetic power from any of these substrates was very much less marked.

Since Corkill and Ochoa had found that glycogen was no longer deposited after an injection of lactate into a 24 hour toxaemic animal, Cross and Holmes tried, with success, to demonstrate glycogen formation in liver slices. The synthesis from lactate and pyruvate was small, that from glucose and still more from fructose, considerably larger. Toxaemia of 24 hours' duration, though it had already been

found to have little effect upon gluconeogenesis, almost completely prevented the formation of glycogen in the slices from lactate or pyruvate, though some glycogen was still formed from glucose. Thus, in *early* toxaemia, it is the power to deposit glycogen rather than to form carbohydrate which the liver seems to lack. If this is so, it follows that, in early toxaemia, an injection of some substance such as lactate which, in the normal animal, is stored in the liver as glycogen, and raises the blood sugar only temporarily or not at all, should give rise to hyperglycaemia. This has been found to be indeed the case (Dawson and Holmes, 1939). As was mentioned earlier, Cross and Holmes also found that addition of a very small quantity of adrenalin to the liver slices inhibited glycogen formation (though not the formation of glucose). It is tempting, therefore, to ascribe the effect to the presence of excess of adrenalin. But in these experiments, glycogen formation from glucose was inhibited by adrenalin as much as was that from lactate, and though such an explanation is in line with some of the earlier work, the objections previously raised against that view remain. It seems probable that toxaemia somehow increases the rate of breakdown of glycogen, rather than that it inhibits its formation, for otherwise it is difficult to account for the fact that in the 24 hour toxaemic animal some glycogen is still formed from injected glucose, but none from injected lactate. If, however, the reversible reaction glucose $\rightleftarrows$ glycogen is shifted to the left in toxaemia, the presence of a high concentration of glucose following glucose injection might still suffice to shift it to the right.

It has become clear from later results (Dawson and Holmes, 1939) that changes besides failure to deposit glycogen take place even after 24 hours' toxaemia, for there is a very marked slowing (as compared with the normal) in the rate at which lactate disappears from the blood stream after it has been injected intravenously, and a large decrease in the rate at which liver slices from toxaemic rabbits, as compared with normal ones, cause lactate to disappear. Yannett and Darrow (1933b) observed a progressive rise in blood lactate during toxaemia. Cross and Holmes (1937), Cross (1938) have compared the oxygen consumption, respiratory quotient, and change in acid-base equilibrium (sometimes known as "glycolysis") of slices of liver from normal, 24 hour toxaemic and 48 hour toxaemic rabbits. As toxaemia increases, the oxygen consumption and respiratory quotients fall. If no substrate is added to the tissue slices, about the same amount of acid is produced after 24 hours' toxaemia as in the normal tissue, but after 48 hours the

acid production is decreased. Lactate and pyruvate added to normal tissue markedly increase the oxygen consumption and raise the R.Q., and acid radicles are removed. It has already been shown that some of it is converted into carbohydrate, and the increased oxygen consumption and raised respiratory quotient show that some of it is burned. In tissue from 24 hour, and still more from 48 hour toxaemic rabbits, the oxygen consumption is decreased, the R.Q. is lowered, and, in the presence of lactate or pyruvate, much less acid disappears than is the case with tissue from normal animals. These observations on isolated tissue are thus in harmony with those on the whole animal, and they indicate that even after 24 hours' toxaemia, still more after 48 hours, there is a disturbance of the oxidation mechanisms as well as of the power to deposit glycogen. It is interesting that the power to form carbohydrate is maintained in 24 hour toxaemia, and is markedly affected only after 48 hours, in spite of the fact that at the earlier stage the oxidation mechanisms are perceptibly damaged, and the rate of disappearance of lactate added to isolated tissue is decreased, as is the rate of disappearance of injected lactate. A further point of interest is that the damaging effect of toxaemia is greater on carbohydrate synthesis from some substrates than from others. Lactate, alanine, and pyruvate form little carbohydrate in the isolated liver after 48 hours' toxaemia, while glycerol, glycerophosphate and glyceric aldehyde still form a good deal (Cross and Holmes, 1937). The oxygen consumption, respiratory quotient, and acid production are only a little less after 48 hours' toxaemia than in normal tissue when glycerol is added as a substrate.[5]

Work of this kind at present involves investigation of the normal mechanisms as well as the disturbances which may occur in toxaemia, and the interpretation of results is often uncertain because the nature of the normal machinery is not sufficiently understood. For instance, the disappearance of lactate (Dawson and Holmes, 1939) and of pyruvate (Cross, 1938) when shaken with isolated liver slices from normal animals is much greater than the disappearance of acid measured

[5] A report has just come to hand of the work of Lyman and Barron (Proc. Am. Soc. Biol. Chemists, p. lxiii, Toronto, 1939). These workers found that the oxidation of pyruvic acid, lactic acid, alanine, aspartic and succinic acids, and choline by the isolated kidney tissue of rats poisoned by diethylene glycol was much diminished when compared with their oxidation by slices of normal rats' kidneys. Ammonia production was also reduced. It was thought that the dehydrogenating enzymes were most affected. Clinically, the animals suffered from a severe nephritis.

manometrically. This means, of course, that lactic and pyruvate acids are converted, during the experiment, into some other acid or acids which have not so far been identified.

Most of the work so far discussed has been concerned with the effects of diphtheria toxin, both in human cases and in animals. That this should have been so is natural, for on the one hand the disease is of great clinical importance, and on the other diphtheria toxin can easily be obtained and standardized, is stable, and has easily reproducible effects when injected into animals; effects, that is to say, which are reproducible within the limits of variation which naturally occur from one animal to another. Making use of an isolated enzyme system, and of toxins from bacteria of a different type, Wooldridge and Higginbotham (1938) have investigated the effect of certain anaerobic organisms—Cl. Welchii, Cl. Oedematiens, Cl. Septique, Cl. Tetani—upon the enzyme system responsible for the oxidation of succinate by extracts of the finely ground small intestines of rats. Positive results were obtained with Cl. Welchii type A., and the authors thought that the "α" toxin was probably responsible. The oxygen uptake of the preparation was definitely inhibited, and the inhibitory effect was reversed both by heating the toxin and by the addition of the appropriate antiserum. Since neither the dehydrogenase nor the indophenol oxidase enzymes appeared to be affected, the authors thought that the toxin probably acted upon some carrier component of the system.

Extremely interesting results have, however, been obtained by the use of non-bacterial toxic agents. Minot (1926) in the course of a study of the effect of carbon tetrachloride administered to dogs found that the substance was particularly lethal to animals kept on a diet low in calcium. High calcium diets exerted considerable protection, and calcium administration relieved the condition. Apparently, the administration did not have much effect on the liver damage, as judged by the histological picture, but did nevertheless save the animals' lives.

Minot and Cutler (1929a, b) found that guanidine, or a guanidine-like substance accumulated in the blood stream of animals suffering from carbon tetrachloride or chloroform poisoning, and of patients with eclampsia or pre-eclamptic toxaemia. Carbon tetrachloride and chloroform produce severe "central necrosis" of the liver, and liver lesions are found in eclampsia (Stander, 1929). Minot and Cutler observed that both experimental animals and patients suffered from hypoglycaemia, and Titus, Dodds and Willetts (1927) record the occurrence of hypoglycaemia in eclampsia. The hypoglycaemia occurring

in animals could be relieved by glucose injections, but calcium administration seemed also to be essential, nor were the nervous symptoms relieved by administration of glucose without calcium. The administration of guanidine (Minot and Cutler, 1929b, c) causes symptoms very like those caused by carbon tetrachloride, which can be relieved by calcium administration. Guanidine retention and calcium reserve are said to act as antagonistic factors in carbon tetrachloride and chloroform poisoning.

Minot (1931) showed that there was a great increase of lactic acid in the blood of dogs poisoned either with carbon tetrachloride or guanidine. In rats, doses of 50 to 75 mgm. of guanidine chloride per 100 grams of body weight caused a fall in blood sugar, a rise in blood lactic acid, a fall in liver glycogen, and a decrease (less marked than in the liver, however) of the total glycogen of the body. These effects were, to a great degree, prevented by calcium administration. In dogs poisoned with carbon tetrachloride, the calcium effect was in turn nullified by the administration of ergotamine tartrate. Minot, Dodd and Saunders (1934) gave repeated doses of 100 mgm. of guanidine hydrochloride subcutaneously to dogs. They found that it gave rise to a severe acidosis, which terminated fatally. The animals could not utilize lactic acid, which accumulated in the blood and was excreted in the urine. There was a fall in the blood CO_2 and bicarbonate. The administration of bicarbonate exacerbated the condition, perhaps by increasing the guanidine concentration in the blood, or by decreasing the ionic calcium. The condition was improved if calcium was given with the alkali.

Further work by Minot and her collaborators has served to emphasize the fact that increases in blood guanidine are often found in toxaemic conditions in which there is serious loss of fluid, such as acute gastroenteritis in infants, and that conversely, guanidine poisoning is associated with dehydration and finally with circulatory collapse (Minot and Dodd, 1933; Minot and Keller, 1936). This action of guanidine on the circulation is counteracted by calcium, and is thought to involve the autonomic system (Minot, 1938a, b).

Minot and Dodd (1933) discuss the toxicology of guanidine, and point out that there is reason to believe that guanidine is liberated during necrosis of tissues.

Marshall and Rowntree (1915) found that there was decreased tolerance to galactose and laevulose in dogs poisoned with phosphorus or chloroform. Althausen and Thoenes (1932a, b) investigated the effects

of phosphorus and chloroform poisoning upon the carbohydrate metabolism of rabbits. Their experiments were rather few in view of the variable behaviour of the animals and the complicated nature of the results. Rabbits were given small doses of phosphorus in oil, injected subcutaneously every day. Their livers underwent fatty degeneration, but if the dose was omitted for a day or two, marked cellular regeneration occurred and a notable degree of resistance was established. Modified glucose tolerance tests were performed. The tests consisted in giving 5 grams of glucose in 100 cc. of water by stomach tube, together with the subcutaneous injection of 1 unit of insulin. These tests were begun 4 days after the first phosphorus injection, and continued daily, the animals feeding well for most of the time.

The administration of the phosphorus caused a diminution of glucose tolerance; in the later stages of the poisoning there was a tendency to marked hypoglycaemia at the end of the test. The hyperglycaemia normally evoked by adrenalin was less than usual. The administration of lactate (2 gm/kilo by stomach tube) caused a hyperglycaemia. After 4 days on phosphorus, adrenalin failed to give a hyperglycaemia, and the liver glycogen was reduced. Glucose and insulin failed to raise the liver glycogen.

Chloroform poisoning (Althausen and Thoenes, 1932b) caused by a single dose of 1 cc. of chloroform given subcutaneously in 33 per cent solution in oil gave rise, in the acute stage, to some hypoglycaemia. There was diminished tolerance to dextrose and insulin, and the hyperglycaemia which normally follows adrenalin injection was diminished, and was sometimes replaced by a hypoglycaemia. During the recovery stage glucose and insulin, or even glucose alone sometimes gave a marked *hypoglycaemia.* The liver glycogen was said to be reduced, but no proper control figures were published.

These experiments are difficult to interpret: the authors were of the opinion that they were dealing with liver damage uncomplicated by other lesions, and it is possible that their results were caused by alterations in the gluconeogenetic and "homoeostatic" mechanisms of the liver. They quote Williamson and Mann (1923), Opie and Alford (1915), and Mosiman and Whipple (1912) as supporting the view that chloroform poisoning causes damage only to the liver. This is quite at variance with what is commonly taught on the subject, and perusal of the papers quoted shows that, while Mosiman and Whipple did express such an opinion, they did not give (in that paper) any experimental evidence for it. Opie and Alford dealt with the effects of diet in liver

lesions caused by phosphorus, chloroform and alcohol and did not investigate any organ other than the liver, while Williamson and Mann are at pains to make it very clear that they do *not* ascribe the death of the majority of their animals after chloroform or phosphorus to liver damage, since only in a few cases was there a hypoglycaemia, rise in blood uric acid, or the appearance of symptoms at all like those following hepatectomy. On the contrary, they insist that death in many cases must have been due to damage to other organs, such as the heart or kidneys.

It is difficult to understand why these authors were so misquoted, nor is it easy to understand why Althausen and Thoenes complicated their experiments by giving insulin as well as glucose and at the same time failed to use more animals or to do a sufficient number of control experiments on the change of liver glycogen.

A very interesting paper on the effects of phosphorus poisoning on carbohydrate metabolism is that of Frank and Isaac (1911). In spite of the fact that they had to use "macro" methods for blood sugar determinations, these authors showed that in rabbits which had received a dose of phosphorus fatal in about 4 days, there was usually a terminal hypoglycaemia. Glucose tolerance tests showed a grossly impaired tolerance, and, again in the later stages, adrenalin failed to give rise to a hyperglycaemia. The authors argue that, in the earlier stages of poisoning, there is a failure on the part of the liver to deposit or retain glycogen. Later, there is a failure of gluconeogenesis, and particularly, they think, in the conversion of lactate to carbohydrate. While they did not bring very much experimental evidence to support these views, they seem, in fact, to fit very well with later findings discussed earlier in this review.

Neubauer (1909) had earlier claimed that after the subcutaneous administration of optically inactive sodium lactate to normal rabbits, there was an increased excretion of other soluble acid in the urine, part only of which was lactic acid. This phenomenon was little affected by doses of phosphorus fatal in about four days. This does not seem to fit very well with the conception of failure in the conversion of lactic acid to carbohydrate as an important feature of phosphorus poisoning.

It seems possible that the discrepancy between the findings of Frank and Isaac and those of Williamson and Mann are to be explained by the fact that the latter authors probably used smaller doses of phosphorus than the former. In their animals the brunt of the effect may have fallen upon organs other than the liver, lesions which were

responsible for the death of the animals, while Frank and Isaac used amounts of phosphorus which quickly caused extensive and fatal liver damage, the effects of which masked those of lesions in other organs.

There is an extensive literature on the effect of phosphorus poisoning upon protein metabolism. To enter into it here would lengthen this review very considerably, and it has been decided, perhaps rather arbitrarily, not to do so. A brief discussion, with many references to the literature, will be found in Lusk's *Science of Nutrition* (1928) and in Marshall and Rowntree's (1915) paper; Frank and Isaac (1911) also refer to a number of the earlier papers.

Hartmann and Senn (1932c) have developed a technique of lactate administration for relief of acidosis in patients (1932a, b). They found that after intravenous (racemic) lactate administration to a group of four patients with catarrhal jaundice ("acute hepatitis") there was a slight but definite delay in the complete metabolism of lactate, as judged by measurement of blood lactate and the CO_2 content of the blood, and a definite hyperglycaemia which, however, disappeared within two hours.

A very similar technique, in which, however, intravenous doses of 50 to 75 mgm. of sodium d-lactate per kilo of body weight were given was used by Stoffer, Dantes, Newburger and Sobotka (1937a). In normal patients, d-lactate injections of this magnitude gave rise to an elevation of blood lactic acid which reached its peak in 5 minutes, and had disappeared within 30 minutes, there was no change in blood CO_2 or blood sugar, and no lactic acid appeared in the urine. They found (1937b) a higher peak in blood lactic acid, and a delayed return to normal in 3 cases of catarrhal jaundice, 4 cases of arsphenamine poisoning, and 1 case of cirrhosis of the liver with superimposed necrosis.

Stoffer, Dantes and Sobotka (1938) applied this test to a group of patients with jaundice with hepatitis, and to another group with jaundice due to extra-hepatic causes. There was delay in return of the blood lactate curve to normal in the former group, but not in the latter. A small group of diabetic patients were also investigated. One only, who had an enlarged liver, showed a delayed curve; but delay in the return of the blood lactate to normal was observed in 3 out of 6 cases of muscular disease.

Stoffer, Dantes and Sobotka (1937) found lesions of the liver and kidneys in dogs with acute arsphenamine poisoning. They state that the other organs were normal, but do not mention any special examination of the endocrine organs. They observed a diuresis, increase in

blood non-protein nitrogen, inorganic phosphate, and lactic acid and a fall in the CO_2 and chloride content of the blood. Three cases showed severe hypoglycaemia. The urinary lactic acid was increased and the urinary chloride and inorganic phosphate diminished; there was albuminuria.

It will be seen from the above discussion that there is a definite parallelism between the changes in carbohydrate metabolism brought about by diphtheria toxaemia on the one hand, and by poisoning by carbon tetrachloride, chloroform, phosphorus, organic arsenicals, guanidine, and apparently in some cases by the toxaemia of eclampsia, on the other. The common features are liver damage, decreased glucose tolerance, low liver glycogen, hypoglycaemia, inability to remove lactic acid from the blood stream; these phenomena may all reflect a diminished power of gluconeogenesis. Cross and Holmes observed (unpublished experiments) that guanidine added to slices in vitro could abolish their power to form carbohydrate. How far, however, the changes all spring from a common cause, only further work will show. Clinically, the conditions just mentioned may be associated with jaundice, although obvious jaundice is certainly not seen in diphtheria occurring either clinically or in experimental animals. So far as we know, no observations have yet been made either on the occurrence of guanidine in the blood in diphtheria or upon the effect of calcium in that condition. The report that ergotamine tartrate prevents the beneficial effect of calcium in experimental carbon tetrachloride poisoning, if confirmed, would suggest that the calcium may act through the autonomic nervous system, perhaps on the suprarenal glands.

There have been fewer observations of the effect of toxaemia upon the metabolism of nitrogen than upon that of carbohydrate. Since it is becoming more and more obvious that, once amino acids have suffered deamination, the fate of their carbon residues becomes indistinguishable from that of three or four carbon chains derived from other sources, it may well become misleading to press the distinction between the effect of toxaemia upon the metabolism of nitrogenous bodies and of carbohydrate or fat. So far as deamination and urea formation are concerned, it is at present difficult to distinguish between the effect of toxaemia upon the liver and the kidney. Kidney damage, or even circulatory failure, may obviously cause a rise in non-protein nitrogen of the blood, by reason of diminished urea excretion; though deamination and urea formation may be normal. On the other hand, since the kidney deaminates very rapidly but contributes a hitherto

unassessed fraction to the total deaminating power of the body, accumulation of amino acids may be partly due to kidney damage as well as to liver damage.

Yannet and Darrow (1933) state that their toxaemic rabbits showed necrosis of the liver parenchyma, a glomerular and tubular nephritis, degeneration of the heart muscle fibres and haemhorrages in the suprarenals. The majority of their animals showed progressive increase both in blood non-protein nitrogen and in blood amino nitrogen. Since the blood amino nitrogen is a comparatively small fraction of the total non-protein nitrogen, there was presumably marked and progressive urea retention which might be attributed to the kidney lesions, the circulatory failure, dehydration (with consequent failure in renal function), or to all three causes combined. It has also been shown that the blood non-protein nitrogen is increased in adrenalectomized animals, so that a rise of blood non-protein nitrogen in a toxaemic animal might be caused by suprarenal damage.

Cross and Holmes (Cross, 1938) found that urea formation from slices of rabbit liver both with and without alanine appeared to be diminished by about 30 per cent of the normal value when the slices were taken from the livers of 48 hour toxaemic animals. The initial values were much higher in the toxaemic animals, and this could well be accounted for by a raised blood urea, which was observed in the cases in which it was looked for

Wakeman and Morrell (1930) found that in experimental yellow fever in monkeys there occurred marked histological lesions of the liver, and often of the kidneys. In the later stages of the disease there was usually a rise in blood non-protein nitrogen, but the amino acid nitrogen was much more increased than the urea nitrogen. This appeared to indicate damage to the deaminating power of the liver or kidneys, or both. The decreased amount of urea might have been due simply to decreased deamination or to a failure in urea synthesis. In the latter case an increase in blood ammonia might have been expected, but was not recorded. Hypoglycaemia was observed.

Marshall and Rowntree (1915) poisoned dogs with phosphorus and chloroform. The N_2 partition in the urine was always disturbed in the direction of increase in amino acid nitrogen and decrease in urea nitrogen. The authors were of the opinion that in phosphorus poisoning, at any rate, renal changes contributed less to the picture than did damage to liver function. In this condition the non-protein nitro-

gen, urea, and amino acid nitrogen was increased both in blood and urine, and this they attributed to increased protein catabolism.

The work which has been reviewed extends over a considerable period of time, and the reports of it are scattered through a number of journals. It is obvious that many of the authors were not aware of the results previously obtained by others. It is easy, however, to follow a common thread which runs through the work. It leads directly to the suggestion that in many different toxaemic conditions the damage falls most heavily upon the machinery of carbohydrate metabolism. At the same time there is thrown into sharp relief the question whether the damage is primarily to endocrine organs which control the normal speed, direction and balance of carbohydrate metabolism, or whether the essential feature is disturbance of those emzyme systems of the cells of the liver and other organs which deal with carbohydrate molecules. There is so much evidence on both sides, that the only safe conclusion at present is that probably both factors come into play in producing the picture of disturbed carbohydrate tolerance, insulin resistance, and finally failure in glyconeogenesis with which we have become familiar. Lines of investigation which should serve further to clarify the problem lie open, and the practical advantages to be gained from such a clarification are obvious, for replacement of deficient endocrine secretions is becoming almost daily more and more within the reach of practical therapeutics.

The trend of modern work on intermediary metabolism may perhaps lead to a simplification of our conception of the effect of toxaemia, for it is becoming plainer and plainer that protein, fat, and carbohydrate all are broken down, during the course of metabolism, to compounds containing three or four carbon atoms, and that once they have reached this stage the products of the main types of foodstuff become indistinguishable. There is strong evidence that it is upon the metabolism of these three and four carbon compounds that toxaemia produces its effect, so that it may well be misleading to think of the effects of toxaemia upon fat or protein metabolism as phenomena distinct from its effect upon carbohydrate metabolism or upon the action of insulin. It may, on the contrary, become both possible and profitable to consider its effect, for instance, upon urea formation or upon the oxidation of pyruvic acid as being essential features of the problem. A "normal" sugar tolerance curve is the result of many factors—an intricate balance of endocrine secretions, the state of the enzyme systems in the various

tissues and the fulness or depletion of carbohydrate stores in the liver and perhaps elsewhere. The observation that the sugar tolerance curve deviates from its normal shape merely tells us that one or more of these conditions is disturbed, and a much closer analysis is necessary before we can describe at all precisely what has happened.

Biochemical findings can with great profit be compared with histological ones. At a crude level this has already been done. For instance Cross and Holmes (Cross, 1938) found that 24 hours after the injection of diphtheria toxin into rabbits there was little to be seen in the way of histological change in sections of the liver, and that while glycogen storage was grossly disturbed, gluconeogenesis was but little affected. After 48 hours' toxaemia necrotic patches were usually obvious in the liver, and gluconeogenesis from lactate and pyruvate were generally much reduced; but in a few of the 48 hour toxaemic animals gluconeogenesis went on as in the normals, and none of these animals showed histological signs of liver damage. The correlation between histological and chemical findings was striking.

Advances in knowledge of pathological conditions must always depend ultimately on clinical observation and study, for only so can the problems be posed and defined; "clinical" here is used in a wide sense, for probably a very great deal more use could be made of veterinary material than is at present the case. But the writer would stress the absolute necessity of combining the use of animal experiment with clinical observation. Without the use of animals, material is difficult to obtain, experimental scope is limited, and adequate controls often impossible. It is true that disease in man is not necessarily—perhaps not often—exactly reproducible in animals. Much of the work here discussed has indeed to do with liver damage during diphtheritic toxaemia, whereas the prominent feature of human diphtheria is circulatory collapse rather than obvious disturbance of liver function. But it is in the highest degree unlikely that the effects of bacterial or other poisons upon fundamentally important enzyme systems will differ from species to species, and it is with such effects that we are primarily concerned. Once these problems have been unravelled, it is likely to be a relatively simple matter to say why the particular manifestations of disease differ from one species to another.

REFERENCES

Althausen, T. L. and E. Thoenes. Arch. Int. Med. **50**: 58, 1932a.
Arch. Int. Med. **50**: 257, 1932b.

Bach, S. J. and E. G. Holmes. Biochem. J. **31**: 89, 1937.

Bamberger, P., H. E. Neuer and H. A. Oelkers. Klin. Wchnschr. **17**: 151, 1938.
Barnes, B. O., V. B. Scott, H. W. Ferrill and J. M. Rogoff. Proc. Soc. exper. Biol. N. Y. **31**: 524, 1933.
Barnes, B. O., A. S. Dix and J. M. Rogoff. Proc. Soc. exper. Biol. N. Y. **31**: 1145, 1933.
Benn, E. C., E. Hughes and S. Alstead. Lancet **i**: 281, 1932.
Bernhardt, H. Deutsch. med. Wchnschr. **62**: 1123, 1936.
Britton, S. W. and H. Silvette. Am. J. Physiol. **107**: 190, 1934.
Buell, M. V., I. A. Anderson and M. B. Strauss. Am. J. Physiol. **116**: 274, 1936.
Buell, M. V., M. B. Strauss and E. C. Andrus. J. Biol. Chem. **98**: 645, 1932.
Cope, O. and H. P. Marks. J. Physiol. **83**: 157, 1934.
Cori, C. F. and Shine. J. Biol. Chem. **114**: xxi, 1936.
Corkill, A. B. J. Physiol, **76**: 381, 1932.
Corkill, A. B. and S. Ochoa. J. Physiol. **82**: 399, 1934.
Cramer, W. Brit. J. exper. Path. **7**: 88, 1926.
Cross, M. C. A. Dissertation for the degree of Ph.D. Cambridge, 1938.
Cross, M. C. A. and E. G. Holmes. Brit. J. exper. Path. **18**: 370, 1937.
Dawson, C. R. and E. G. Holmes. In press, 1939.
Deuel, H. J., L. F. Hallman, S. A. Murray and L. I. Samuels. J. Biol. Chem. **119**: 607, 1937.
Dodd, K., A. S. Minot and H. Casparis. Am. J. Dis. Child. **43**: 1, 1932.
Edmunds, C. W. and F. D. Johnston. J. A. M. A. **90**: 441, 1928.
Elkeles, A. and F. Heimann. Deutsch. Arch. Klin. Med. **155**: 263, 1927a.
Deutsch. Arch. Klin. Med. **158**: 238, 1927b.
Elliott, T. R. J. Physiol. **44**: 374, 1912.
Evans, C. L. and I. Zweckwer. Brit. J. exper. Path. **8**: 280, 1927.
Frank, E. and S. Isaac. Arch. exper. Path. u. Pharmakol. **64**: 274, 1931.
Gemill, C. L. and E. G. Holmes. Biochem. J. **29**: 338, 1935.
Hanzlik, P. J. and B. Terada. J. Pharmacol. **56**: 269, 1936.
Harde, E. and H. R. Benjamin. Proc. Soc. exper. Biol. N. Y. **32(1)**: 651, 1935.
Harding. Lancet **200**: 737, 1921.
Harris, L. J., R. Passmore and W. Pagel. Lancet **ii**: 183, 1937.
Hartmann, A. F. and M. J. E. Senn. J. Clin. Investigation **11**: 327, 1932a.
J. Clin. Investigation **11**: 337, 1932b.
J. Clin. Investigation **11**: 345, 1932c.
Hartmann, F. A. and W. J. M. Scott. Proc. Soc. exper. Biol. N. Y. **28**: 478, 1930.
Hector, F. J. Lancet **ii**: 642, 1926.
Herbrand. Klin. Wchnschr. **14**: 623, 1935. Fortschritte der Therapie **13**: 655, 1937.
Hibbard, C. L. and M. J. Morrissey. J. exper. Med. **4**: 137, 1899.
Himsworth, H. P. J. Physiol. **81**: 29, 1934.
Lancet **ii**: 935, 1932.
Proc. Royal Soc. Med. **29**: 658, 1936.
Himsworth, H. P. and Scott. J. Physiol. **92**: 183, 1938.
Hoet, J. Reprint of a Communication to the Congres National des Sciences Belgium.

Ingle, D. J. Am. J. Physiol. **116**: 622, 1936.
Jeney, A. V., J. Gagyi and P. Baranyai. Deutsch. med. Wchnschr. **62**: 54, 1936.
Jungeblut, C. W. and R. L. Zwemer. Proc. Soc. exper. Biol. N. Y. **32**: 1229, 1935.
King, C. G. and M. L. Menten. J. Nutrition **10**: 129, 1935.
Kotliarov, I. I. Bull. de Biologie et de Medecine exper. de l'U.S.S.R. **6**: 475, 1938.
Lawrence, R. D. and O. B. Buckley. Brit. J. exper. Path. **8**: 58, 1927.
Lotze, H. and S. Thaddea. Virchow's Arch. **300**: 685, 1937.
Lusk, G. Science of nutrition. 4th ed. W. B. Saunders Co., Philadelphia & London, 1928.
Lyman, C. M. and C. G. King. J. Pharmacol. **56**: 209, 1936.
Mann, F. C. and J. Bollmann. Am. J. Physiol. **110**: 45, 1935.
Marks, H. P. Proc. Royal Soc. Med. **29**: 663, 1936.
Marshall, E. K., Jr. and L. G. Rowntree. J. exper. Med. **22**: 333, 1915.
Menten, M. L. and H. M. Manning. J. Med. Res. **44**: 675, 1924.
Messer, H. Deutsch. med. Wchnschr. **62**: 1131, 1936.
Mikami, S. T. J. exper. Med. **6**: 299, 1925.
Minot, A. S. Proc. Soc. exper. Biol. N. Y. **24**: 617, 1926.
J. Pharmacol. **43**: 295, 1931.
Minot, A. S. and J. T. Cutler. Proc. Soc. exper. Biol. N. Y. **26**: 607, 1929a.
J. Clin. Investigation **6**: 369, 1929b.
Proc. Soc. exper. Biol. N. Y. **26**: 138, 1929c.
Minot, A. S., K. Dodd and J. M. Saunders. J. Clin. Investigation **13**: 917, 1934.
Minot, A. S. and K, Dodd. Am. J. Dis. Child. **46**: 522, 1933.
Minot, A. S. and M. Keller. J. Pharmacol. **60**: 32, 45, 1936.
Minot, A. S. J. Pharmacol. **65**: 243, 1938a.
J. Pharmacol. **65**: 253, 1938b.
Mosiman, R. E. and G. H. Whipple. Johns Hopkins Hosp. Bull. **23**: 323, 1912.
Myers, G. N. J. Pharmacol. **49**: 483, 1933.
Neubauer, E. Arch. exper. Path. und Pharmakol. **61**: 387, 1909.
Opie, E. L. and L. B. Alford. J. exper. Med. **21**: 1, 1915.
Perla, D. and J. Marmorsten-Gottesman. Proc. Soc. exper. Biol. N. Y. **28**: 475, 1930a.
Proc. Soc. exper. Biol. N. Y. **28**: 650, 1931b.
Rosenthal, F. Arch. exper. Path. und Pharmakol. **78**: 99, 1914.
Schwenkter, F. and W. W. Noel. Johns Hopkins Hosp. Bull. **46**: 259, 1930a.
Johns Hopkins Hosp. Bull. **46**: 359, 1930b.
Scott, W. J. M. and W. L. Bradford. Proc. Soc. exper. Biol. N. Y. **28**: 428, 1930.
Sigal, A. and C. G. King. J. Pharmacol. **61**: 1, 1937a.
J. Pharmacol. **59**: 468, 1937b.
Soskin, S. and M. D. Allweiss. Am. J. Physiol. **110**: 4, 1934.
Soskin, S., M. D. Allweiss and D. J. Cohen. Am. J. Physiol. **109**: 55, 1934.
Soskin, S., M. D. Allweiss and I. A. Mirsky. Arch. Intern. Med. **56**: 927, 1935.
Soskin, S., H. E. Essex, J. F. Herrick and F. C. Mann. Am. J. Physiol. **124**: 558, 1939.
Soskin, S. and I. A. Mirsky. Am. J. Physiol. **112**: 649, 1935.
Stander, H. J. Medicine **8**: 1, 1929.

Stoffer, L. J., D. A. Dantes, R. Newburger and H. Sobotka. Arch. Int. Med. 60: 876, 1937a.
Arch. Int. Med. 60: 882, 1937b.
Stoffer, L. J., D. A. Dantes and H. Sobotka. Arch. Int. Med. 60: 509, 1937.
Arch. Int. Med. 62: 918, 1938.
Sweeney, J. S. Arch. Int. Med. 41: 420, 1928.
Sweeney, J. S., N. Barshop and L. C. LoBello. Arch. Int. Med. 53: 689, 1934.
Sweeney, J. S., N. Barshop, L. C. LoBello and R. J. Rosenthal. Arch. Int. Med. 54: 381, 1934.
Sweeney, J. S. and R. W. Lackey. Arch. Int. Med. 41: 257, 1928.
Thaddea, S. Klin. Wchnschr. 2: 1275, 1935.
Dtsch. med. Wchnschr. 62: 1117, 1936a.
Dtsch. med. Wchnschr. 62: 1171, 1936b.
Titus, P., P. Dodds and E. W. Willetts. Am. J. Obstet. and Gynaec. 14: 89, 1927.
Torrance, C. C. J. Biol. Chem. 121: 31, 1937.
Wakeman, A. M. and C. A. Morrell. Arch. Int. Med. 46: 290, 1930
Williams, J. L. and G. F. Dick. Arch. Int. Med. 50: 801, 1932.
Williamson, C. S. and F. C. Mann. Am. J. Physiol. 65: 267, 1923.
Wooldridge, W. R. and C. Higginbotham. Biochem. J. 32: 1719, 1938.
Yannet, H. and D. C. Darrow. J. Clin. Investigation 12: 767, 779, 1933a, b.
Yannet, H. and W. Goldfarb. J. Clin. Investigation 12: 787, 1933.
Young, F. G. Biochem. J. 32: 1521, 1938a.
Biochem. J. 32: 524, 1938b.
Zilva, S. S. Brit. J. exper. Path. 18: 449, 1937.
Zunz, E. and J. La Barre. Arch. Int. Physiol. 29: 265, 1927.
Zweckwer, L. T. and H. Goodell. J. exper. Med. 42: 43, 1925a.
J. exper. Med. 42: 57, 1925b.
Zwemer, R. L. and C. W. Jungeblut. Proc. Soc. exper. Biol. N. Y. 32: 1583, 1935.

THE PRESENT STATUS OF THE BARBITURATE PROBLEM

A. L. TATUM

Department of Pharmacology, University of Wisconsin, Madison

The subject assigned to the writer for discussion is so wide in scope that it seems feasible to attempt only a broad survey, the primary objective being to furnish leads to various aspects of the barbiturate[1] problem as well as to supply a certain amount of useful information.[2]

Brain. The brain is the chief site of action in that hypnosis and amnesia, and in overdosage, anesthesia and coma, are the commonly expected dominant effects. Masserman (113), on the basis of electrical stimulation of the anterior hypothalamus, found that barbiturates (amytal) caused a depression or diminution of reactivity of this region, while after small doses, the motor cortex was relatively unaffected. On the other hand, Fulton and Keller (43) saw depression of the cortex after nembutal (pentobarbital) prior to depression of the hypothalamic centers and explain, on this basis, the period of excitement so often seen in laboratory animals.

On the basis of a study of regions of action of certain relatively effective analeptics, indirect evidences also point to the basal ganglia and hypothalamus as being the regions of greatest susceptibility (111). On account of relatively specific functional susceptibility of certain cells to appropriate chemical agents, chemical recovery of these agents leads to equivocal results. The Keesers (78, 79) claim to have demonstrated a higher concentration of barbiturates in the hypothalamus than in other parts of the brain. Koppanyi and Dille (92), however, claim that barbital occurs in the same concentration here as in other parts. Thus, the determination of points or regions selectively affected appears to require functional rather than chemical evidences.

Schriever and Perschmann (155, 156) infer that barbiturates act upon the basal ganglia and hypothalamus owing to the effective antag-

[1] The reviewer uses the term "bar'biturate" (pronounced most euphoniously with accent on the first syllable) in the generic sense, even including the acid form.

[2] For a review of the literature prior to 1929, see Lundy and Osterberg (104).

onism by picrotoxin and the relative localization of the latter drug to that region of the cerebrospinal axis.

Respiration. Little can be said as to the direct action of barbiturates on the medulla since such common phenomena as dominant respiratory depression and paralysis in overdosages may well be referable to effects of the drugs on higher centers. Complete but temporary respiratory paralysis can be readily demonstrated to occur in laboratory animals or man with relatively little effect on the vasomotor center, as indicated by negligible changes in blood pressure. Marshall et al. (111) explain the antagonism of barbiturate depression by picrotoxin as a decreased narcosis rather than stimulation of the respiratory center depressed by the hypnotic agent. On the other hand, Marshall and Rosenfeld (110) differentiate depression of the respiratory center from depression of the carotid sinus mechanism. Barbiturates cause a depression of the respiratory center, the activity of which, however, may be maintained by oxygen-want operating upon and through the sino-aortic mechanism. Free oxygen supply, by diminishing oxygen-want, may cause the appearance of greater depression. Thus, barbiturates appear to depress the central respiratory center mechanisms more than the sino-aortic mechanism. Urethane, paraldehyde and alcohol appear to depress, apparently equally, both the sino-aortic mechanism and the respiratory centers.

Burstein and Rovenstine (14) have demonstrated that short and ultra-short-acting barbiturates cause adduction of the vocal cords in cats, a phenomenon frequently seen also in man. This adduction of the vocal cords is abolished by atropine; hence they conclude that the adduction reflex, under parasympathetic innervation, is stimulated by barbiturates, or at least retained in apparently exaggerated form. It may be that this apparent stimulation is but a manifestation of release from control by higher centers or that this particular reflex is especially difficult to suppress.

Circulation. 1. *Vagus.* De Waele (26) reported vagus nerve depression from the effects of the mixture known as "Somnifene." In 1929, Lieb and Mulinos (101) called attention to a depressant or paralytic action on the cardiac vagus mechanism of the rabbit, cat and dog, by amytal. Garry (44) confirmed the observations of Lieb and Mulinos, and added the conclusion that amytal depresses peripheral vagal ganglia. Acetylcholine continues to be active during the period of amytal action; hence he concluded that the post-ganglionic fibers of the vagus are still responsive. Thus, under the conditions of his experi-

mentation, the action of amytal is largely limited to the ganglia. Garry, furthermore, called attention to differences in different barbiturates, in that amytal was effective on the vagus, whereas in ordinary doses phenobarbital was not. These conclusions appear to be confirmed by the work of Koppanyi, Linegar and Dille (93) who demonstrated that both pilocarpine and physostigmine are effective on the cardiac vagus mechanism even though barbiturates have blocked out the effectiveness of electrical stimulation of the vagus trunk. These authors also demonstrated that the effectiveness of different barbiturates differs considerably in that amytal, pentobarbital and pernoston are effective in moderate doses whereas barbital was effective only when dosages were relatively enormous. They did not succeed in demonstrating this depressant effect with phenobarbital in any dosage whatsoever.

Swanson and Shonle (167) failed to demonstrate a vagal depression after pentobarbital, which is likely due to the use of inadequate dosages. It would seem that among the various barbiturates amytal is one of the most effective vagus nerve depressants while phenobarbital is one of the least effective.

Gruber et al. (56) found that thiobarbiturates, if they affect the vagus at all, cause a sensitization of the vagus to electrical stimulation, a phenomenon seen by De Waele, in the early phase of action of "Somnifene." Thus, it may be possible that the sensitization of the vagus by thiobarbiturates is the only manifestation developed by these derivatives owing to the very brief period of their action in contrast to the longer-acting barbiturates which give ample time for the development of depressant effects.

In cold-blooded animals, Gruber et al. (57) have demonstrated that barbiturates, when applied directly to the heart, cause not only paralysis of ganglia, but also nerve endings as well. Possibly differences in local concentrations account for the divergence of views of Gruber and other workers.

2. *Heart.* Roth (150) observed that the hearts of elasmobranchs are stopped by 0.05 per cent solution of pentobarbital, amytal and luminal, whereas barbital failed to cause stoppage. Perfusion of the rat heart by M/500 solutions of each of several barbiturates caused depression (151). Johnston (73) rates the effectiveness of equal concentration of a number of barbiturates in decreasing the amplitude of contraction of the turtle heart, as follows: ortal, amytal, pentobarbital, evipal, phenobarbital, phanodorn, barbital. It will be noted that this order is not strictly parallel to the order of central nervous system toxic-

ity, as indicated by depression or paralysis of the respiratory center (see classification). Johnston (74) demonstrated, furthermore, that barbiturate poisoning of the isolated turtle heart is very difficult to antagonize, the most effective agents tried being glycine and xanthine, which were not good, whereas picrotoxin, metrazol and coramine were poor. Hafkesbring and MacCalmont (59) reported on the electrocardiograms obtained on dogs and cats under the influence of each of the following: nembutal, amytal and barbital. The only cardiac effects of anesthetic doses of each of these agents as shown by the electrocardiogram were an increase in heart rate and a decrease in the common sinus arrhythmia seen in dogs and cats.

Gruber et al. (53, 55) have reported that anesthetic doses of some thiobarbiturates resulted in alternate ventricular rhythm in dogs, rabbits, cats and monkeys. In dogs the P-R interval is shortened. Stimulation of the peripheral vagus may induce an arrhythmia if it had not been present, but if present, vagus stimulation may cause return to normal rhythm. While atropine has no effect on the arrhythmia due to thiobarbiturates, epinephrine may restore the rhythm to normal. Agents that lower blood pressure are also likely to restore the rhythm to normal. Kohn and Lederer (85) studied the EKG in dogs, cats, rabbits and monkeys receiving pentothal and only occasionally did they see abnormal rhythms due to the drug. Ephedrine occasionally restored the rhythm in those few instances in which an abnormal rhythm occurred. In all cases the abnormal rhythm disappeared on termination of anesthesia.

Betlach (5) reported on the EKG in dogs under the influence of amytal and of pentothal and observed no significant alterations. He insists that dogs are so commonly subject to abnormal rhythms that months of observations must be made on each individual animal before drugs are tried in order to know whether or not any irregularities are really due to specific effects of the agents employed. On the clinical side, Volpitto and Marangoni (179) carefully observed seventeen clinical cases of pentothal, thioethamyl and evipal anesthesia and observed no deviation whatsoever from the normal EKG. Cardiac irregularities already present were not altered by these drugs.

It is obvious, therefore, that the question of cardiac irregularities is still open to question and that while they may be seen occasionally in laboratory animals under the influence of thiobarbiturates, the cautions suggested by Betlach and also by Meek and Seevers (115) must be very seriously considered before one is justified in ascribing irregularities

to any one etiological factor, particularly new drugs. To date, therefore, Betlach and also Volpitto and Marangoni appear to have the best of the argument, and until further observations to the contrary have been made, one must conclude that in man, at any rate, no significant alterations in heart action can be ascribed to the specific activity of either barbiturates or their thio-derivatives.

3. *Vasomotor mechanisms.* Bouckaert and Heymans (12) demonstrate a suppression of presso-receptor vasomotor reflexes in connection with the carotid sinus. Nowak (128) claims that amytal and evipal depress the sino-aortic reflexes somewhat less than corresponding doses of pernoston.

In the regulation of vasomotor tone, Nowak and also Bouckaert and Heymans call attention to the varied effects of different barbiturates in depressing the sino-aortic reflex mechanism. Numal and pernoston appear to depress this vasomotor-controlling mechanism more than do amytal and evipal. Thus we have further evidences relative to the different potentialities of the different barbiturates in their modification of activity of peripheral mechanisms. Both the vagus in its peripheral cardiac action and the sino-aortic mechanism in its reflex action are modified differently by different members of the series.

4. *Blood vessels.* Gruber and Baskett (47), and later Gruber and Roberts (48), reported that phenobarbital, administered to laboratory animals under ether anesthesia, caused a drop in blood pressure. Earlier, Tatum and Parsons (171) called attention to the utility of barbital as an anesthetic for dogs since it had the significant property of preserving an approximately normal blood pressure. The inconsistency in reports of these two groups of investigators illustrates how both parties may be right in their observations and yet appear to disagree because of differences in circumstances. According to Bleckwenn (8), barbiturates, acutely administered, generally cause a drop in blood pressure in both laboratory animals and in man. He largely overcame this difficulty clinically by a very slow administration. In the reviewer's laboratory, it has been found that after a lapse of an hour or more the blood pressure becomes stabilized at a level essentially normal. It is not to be forgotten that there are marked individual differences in both man and laboratory animals.

Nomura (127) found the intestinal blood vessels to be dilated, both *in vivo* and *in vitro*. Swanson and Shonle (166) support Bleckwenn to the effect that the fall in blood pressure can be avoided to a very considerable extent by reduction in the rate of injection. Werner (183), in

studying two ultrashort-acting barbiturates, observed that evipal was more prone to produce an acute fall in blood pressure than was pentothal when given to rabbits by intravenous injection in equivalent anesthetic doses. Marx (112) called attention to increased permeability of blood vessels, which may be a factor in causation of cerebral edema so often seen in serious human poisonings by these drugs.

The drop in blood pressure is obviously due to vasodilatation, even demonstrable in perfusion experiments on the brain of the dog. Horsley (70) has observed an abrupt fall in cerebrospinal pressure succeeded by a slow rise. Gruber and Roberts (48) state that barbiturates effect a coronary dilatation when perfused through the cat or rabbit heart.

Gruhzit et al. (58) state that thiobarbiturates are especially prone to produce congestion, stasis, and even hemorrhage in lungs, liver, kidney and the central nervous system. However, clinical experiences with thiobarbiturates indicate that, in a practical way, these manifestations are rarely encountered. This may be due to the fact that man is far easier to anesthetize than laboratory animals, hence requires much smaller doses. Yet the observations of Gruhzit et al. might well be expected to be seen in man in instances of heavy overdosage providing that the patient survives long enough for these manifestations to develop.

Spinal cord. Direct effects on the spinal cord are clearly demonstrated by Dawson and Porter (23) and by Porter and Allamon (133) in their study of the spinal cat. Such preparations receiving evipal, amytal, barbital or luminal reveal a markedly raised threshold of flexion reflexes. Lowering of the threshold by strychnine can be effectively overcome by barbiturates, even to the extent of practical elimination of all crossed reflexes.

Secretory nerves. Stavraky (164) states that amytal, and less readily dial, depress or paralyze the chorda tympani nerve in the dog. Electrical stimulation and also acetylcholine fail to provoke the expected secretion, as after light ether anesthesia, but pilocarpine effects a copious secretion while physostigmine reëstablishes the efficiency of electrical stimulation. These results would lead one to consider amytal to have affected the secretory nerve endings rather than ganglia, adding evidence to Gruber's contention that amytal actually does affect these endings, possibly in addition to ganglionic depression.

Smooth muscle. 1. *Gastrointestinal muscle.* Gruber (49) demonstrated that all of a number of barbiturates studied cause a depression of intestinal, uterine and ureteral muscle *in vitro.* Later, he and asso-

ciates (51) observed a lowering of intestinal tone in Thiry-Vella loops in non-anesthetized dogs to which phenobarbital had been administered. Reynolds (143) observed also a depression of smooth muscle *in vitro* on use of relatively strong solutions of barbiturates, whereas he observed manifestations of stimulation with very dilute solutions. Gruber et al. (52) rather clearly demonstrated that barbiturates in any effective concentration produce depression and that Reynolds' observations of a stimulant action were confirmed only when the alkalinity was allowed to rise. The so-called stimulant dilutions did not produce stimulation if the pH of the solutions was carefully controlled. It is thus obvious that any stimulant action *in vitro* due to alkalinity of strong solutions is overbalanced by the depressant action of the drugs. In dilute solutions the effects are largely, if not entirely, due to alkalinity.

Quigley and Phelps (138) observed that barbiturates produced inhibition of gastric muscle tone equally in normal dogs and in dogs with denervated stomachs; hence the depression is peripheral either on smooth muscle directly or on the peripheral neuromuscular mechanism. This means that the concentrations of the drugs *in vivo* are of such an order of magnitude as to resemble the effects of these drugs *in vitro.* Burstein (15) very recently called attention to a primary depressant action of short-acting barbiturates *in vivo* followed by a prolonged increase in contractions and tonus. Whether this secondary phase is due to a positive stimulant action or is of a secondary nature was not established.

2. *Bladder.* Kolb and Langworthy (88) observed that the barbiturates depress the urinary bladder stretch reflexes of the cat and prevent a sustained vesicle contraction when filling is complete.

3. *Ureters.* Gruber (49, 50) demonstrated a depression of irritability and peristaltic activity of ureters *in vitro.*

4. *Uterus.* Gruber et al. (49, 52) and Boyd (13) observed that barbiturates depressed uterine strips *in vitro.* Berutti (4) found that both pregnant and nonpregnant uteri of rabbits were depressed *in vitro* by relatively strong solutions but unaffected by weaker solutions.

5. *Placental permeability.* Since the placenta is permeated by most crystalloids and many colloidal substances, it is not at all surprising that barbiturates can be demonstrated in the fetus, as reported by Fabre (37) and by Dille (27).

Kidney action. Ogden (130) called attention to the action of amytal in inhibiting water diuresis in dogs during the period of anesthesia. Walton (181) and Gouaux, Cordill and Eaton (46), in studying the 24-

hour urine output, also total blood and urea nitrogen, observed no significant effects of amytal. Any suppression of activity during the short periods of anesthesia was entirely compensated for by a subsequent and transient period of increased action. The kidney function is thus not seriously affected, provided the period of deep depression is not long. If depression is of long duration, partial inhibition of renal action might be expected to be of greater consequence. It is generally recognized that in clinical cases of severe intoxication secretion of urine is apt to be scanty (34).

Blood. In general, the constitution of blood is relatively little affected by the barbiturates when given in moderate dosages. In the rat, which has a much larger relative spleen weight than have other animals, amytal appears to cause a significant acute enlargement of the spleen with an accumulation of erythrocytes, thus withdrawing them from the blood stream, leading to a dilution of the blood. Leucocytes, on the contrary, are apt to increase in number. Splenectomy prevents these changes in blood cell counts (35). Elman, Weiner and Cole (33) observed that the expected dilution of the blood as a consequence of hemorrhage does not occur when the animals are under the influence of amytal. This may be interpreted to mean that amytal causes an accumulation of red cells within the spleen with a dilution of blood. Hemorrhage removes diluted blood. In the course of a short time, red cells return to the circulating blood, thus counteracting the expected dilution due to hemorrhage.

It is generally held that blood sugar is not changed significantly by barbiturates unless depression reaches such a grade as to bring on acidosis. Alkali reserve (CO_2 capacity) and CO_2 content have been said to increase (139), but probably simply due to the alkalinity of the soluble barbiturate. There does not appear to be any significant direct change in CO_2 capacity or CO_2 content other than may be expected from added alkali or by an acidosis from profound depression, involving as it does the respiratory and other centers. Dorfman and Brooks (29) associate their observations of increase in sedimentation rate with dilution of blood due to barbiturates, which condition would indicate some changes in blood viscosity scarcely accounted for by red cell removal.

Metabolism. Anderson et al. (1) have reported their observations on the effects of different barbiturates on basal metabolism. According to them, barbital, ipral, neonal and phanodorn in "usual therapeutic doses" cause some depression of oxygen consumption, whereas amytal and phenobarbital increase it. Shapiro (159), on the other hand,

states that amytal, in intravenous dosages sufficient to produce corneal anesthesia, caused a profound lowering of oxygen consumption. These results are confirmatory of the experiences of Dameshek et al. (22) who ascribed the fall in oxygen consumption to an action on the hypothalamus. Siebert and Thurston (162) observed that amytal, in dosage adequate to "quiet" the animals, produced no significant change in basal metabolic rate. Thus we are forced to believe that the discrepancies between different observers are simply due to differences in dosage and that, in light hypnotic doses, no significant depression below the resting stage occurs, whereas if a more profound depression occurs, a fall in basal metabolic rate is entirely in line with expectations. In the *in vitro* study of barbital on the oxidation-reduction system of methylene blue-ground muscle tissue, Behnecke (3) demonstrated a reduction of oxygen consumption. Other more or less parallel work has been reported by Quastel and Wheatley (137), Jowett and Quastel (76), and Jowett (77). It is yet too early to extend these results to central effects of barbiturates so far as concerns mechanism of action.

Elimination and detoxication. In usual therapeutic dosages, barbiturates are for the most part destroyed in the body of mammals, and only barbital and luminal appear consistently in the urine. Herwick (62, 63) and Shonle et al. (160) demonstrated that pentobarbital, amytal and neonal are not excreted in the urine by dogs receiving amounts causing readily tolerable depression. Koppanyi and his associates (91), however, claim that by the use of the cobalt color reaction all barbiturates can be detected in the urine of animals receiving these drugs. The disagreement between Koppanyi and other investigators appears to reside in either the nonspecificity of the cobalt color reaction or that the dosages were widely different. Both groups of investigators will agree that in dangerously large doses most of the barbiturates will appear in the urine; hence it is likely that there exists a renal threshold for barbiturates. Thus, if the concentration in the blood be low, most of them do not pass through, whereas if the concentration be relatively high, most of them may be demonstrated in the urine. There appears to be some correlation between the stability of the individual barbiturates and their excretability under conditions of moderate dosage. The duration of action may be used as a biological test of stability and, on this basis, barbital, phenobarbital and dial appear to be relatively stable and long acting and appear in the urine in detectable and measurable quantities. On the other hand, those members of the series which on the basis of a relatively short period of action are more unstable, appear

in the urine only under unusual circumstances such as in instances of heavy dosage. A different situation was observed by Pratt (134) who demonstrated that in instances of injury to the liver, e.g., by chloroform or carbon tetrachloride, the short-acting or more unstable barbiturates become longer acting. These conclusions were essentially confirmed by Koppanyi et al. (94) and by Cameron and de Saram (18). Hirschfelder and Haury (65) observed that double nephrectomy had little if any effect on duration of short-acting barbiturates whereas barbital depression was continued until terminus due to the consequences of nephrectomy. Koppanyi et al. (89, 121, 122) reported similarly and added the observation that renal damage through the action of uranium or chromates caused a distinct retardation of excretion of barbital and little change in the course of depression by the destructible barbiturates. These authors also observed that birds and turtles appear to be unable to excrete barbital effectively; hence deep depression due to barbital is permanent whereas recovery from short-acting or destructible barbiturates does occur. Tyson et al. (175) report that concentrated doses of barbiturates (phenobarbital) may be excreted in part also by the mammary glands. If doses be small and repeated, they do not appear consistently in the milk, thus indicating an excretory threshold more or less analogous to renal thresholds.

Kohn and Grimes (86) have made a study of destruction rate of pentobarbital *in vivo* by functional methods which should be similarly applicable to other barbiturates. The method essentially involves determinations of quantities of a barbiturate required to cause death after the lapse of various time intervals after an initial dose. This indirect method is likely to yield data fairly closely paralleling that obtained by complete extraction of carcasses of animals sacrificed at various time intervals after drug administration. Whether or not destruction rates decline in animals in a moribund state or in profound depression has not been clearly established. Koppanyi et al. (95) state that resuscitation of depressed animals by means of stimulant analeptics leads to no significant changes in barbiturate content of the blood and organs of the stimulated animals when compared to the more depressed controls. Thus, the problem of possible changes in rate of destruction according to conditional states has yet to be answered except in instances of parenchymatous damage such as are effected by chloroform or other poisons which particularly affect the liver.

Syncrgism or summation. Stormont et al. (165) presented convincing evidence that barbiturates significantly increased the anesthetic poten-

tiality of nitrous oxide. With rats as test objects they stated that 85 per cent N_2O-15 per cent O_2 was not anesthetic. When a parallel set of observations was made on rats receiving from 27.5 to 45 per cent of the M.L.D. of barbiturate (dial, neonal, phenobarbital, amytal or barbital), the gas-mixture given to the previous series became effective. Isenberger (71) found that amytal usually considerably reduced the amount of general anesthetic necessary as well as the concentration required for complete anesthesia and relaxation. Kleindorfer and Halsey (82) found that the extinction of the crossed reflex could be accomplished with lower concentrations of ethylene or nitrous oxide when either of these gases was given to animals which had previously received either amytal, dial or allyl-isopropyl barbituric acid. Halsey and Lacey (60) reported experiments on dogs with light ether anesthesia maintained constant by closed system control. When such animals were given either dial or pentobarbital profound anesthesia resulted. If the ether concentration was maintained constant, the animals receiving pentobarbital recovered to a light stage of anesthesia (i.e., ether acting alone) much sooner than those receiving dial. By sharp contrast Calderone (17), in studying the same problem but controlling the blood concentration of ether, came to the conclusion that amytal caused no modification or change whatever in the concentration of ether in the blood requisite to produce any desired stage of anesthesia. Robbins et al. (147), studying cyclopropane, observed that barbital or amytal reduced the concentration of the gas necessary for anesthesia or respiratory arrest. Thus, Calderone appears to stand alone in his contention that pre-anesthetic medication by a number of agents, including barbiturates, does not diminish the amount or concentration of anesthetics required. Unless ether is radically different from cyclopropane the clear-cut report of Robbins et al. is quite convincing that their conclusions and generalizations therefrom are correct and consistent with the practically unanimous opinion of all clinical anesthetists. Possibly some unrecognized analytical error led Calderone to come to his heterodox conclusions. It is too much to expect, with substances as different in action as anesthetics and hypnotics, that addition would be complete. Each substance may well have a characteristic cerebral area of action. Two different substances may have mosaic patterns which overlap to some degree, hence some additive action. Complete overlapping would thus mean identical effects, and identical effects would only be produced by identical substances. Hesse, Baumgart and Dickmann (64) illustrate this point in a quite striking manner when

they demonstrate the potentiation of barbital by phenacetin. Phenacetin is not a hypnotic drug; yet when given with a barbiturate, the effects are clearly accentuated. Phenacetin may be considered to have several "points" of action, one of which is the "sleep center," which is so insignificantly affected as to be unrecognized. Yet when a true hypnotic is simultaneously employed, the points affected in common by the antipyretic and the hypnotic drugs are more affected than would be the case by either acting alone.

Other examples of synergism are reported. Robinson (148) states that calcium potentiates the action of phenobarbital; De Nito (25), that acetylcholine potentiates the action of both magnesium and barbital; Dille and Ahlquist (28), that alcohol increases the action of pentobarbital.

Perhaps the most important practical example of synergism or coaction is the well-known potentiation of barbiturates by morphine. Lundy (105) stated that morphine prolongs the depression produced by amytal. Rowbotham (153) makes the statement that morphine very greatly increases the depressant action of barbiturates. Seevers (158) utilized this principle in using morphine a half-hour or so prior to intravenous administration of pentobarbital for surgical anesthesia in dogs. He found that satisfactory surgical anesthesia could be obtained by the administration of approximately one-half of the dose of pentobarbital that would have been required had morphine not been used. Recovery was quite satisfactory. Vogeler and Kotzoglu (177) used morphine as a predmedication agent prior to use of evipal in rabbits; 100 mgm. per kilogram of morphine one-half hour before caused evipal (20 mgm. per kilogram) to produce a depression lasting sixty minutes, whereas the same dose of evipal without morphine produced a depression lasting for six minutes. Redenz (141) reports confirmation of these results. Pertinent evidence was reported also by Kozelka et al. (98) to the effect that very small doses of morphine given to rabbits very clearly and significantly retarded the destruction of amytal *in vivo*. The cause of this delayed destruction was not established but was assumed to be due to altered circulation of blood through the liver and other abdominal viscera.

Most investigators recognize that when barbital is used as a laboratory anesthetic for dogs (171), a rather long induction period follows even an intravenous injection. In the reviewer's classroom laboratory this inconvenience has been overcome by Seevers and Werner (personal

communication) by the simultaneous administration of pentothal.[3] The animal is anesthetized immediately and continues in this state quite smoothly for hours. Since pentothal alone, in the doses employed, would produce depression for a matter of minutes, a period shorter than the latent period of barbital, it is surprising that the gap is so satisfactorily and evenly bridged.

Antagonism. A study of this phase of the problem has been the subject of a considerable number of contributions, both from the point of view of mechanism as well as practical utility in exigencies as they may arise in clinical practice. Many substances have been used as antagonists to barbiturates in the several manifestations of their actions. In the experimental field, the work of Maloney et al. (108) called attention to the superior efficacy of picrotoxin as a stimulating analeptic. This agent previously had been relegated to the group of outmoded drugs, but in a careful survey these authors observed a superior potency in counteracting the depression effected by barbiturates. Most subsequent investigators have confirmed the observations of Maloney et al. on picrotoxin, especially in more serious grades of hypnotic drug depression. Coramine was recommended by Reese (142) who found that in mild clinical depression it was an effective agent. Caffeine was practically equally effective. Experimentally, coramine as well as caffeine is capable of arousal or partial arousal in animals not profoundly depressed. In the case of deep depression these agents are followed by added depression, and hence are not appropriate to the antagonism in question if recovery is expected. Metrazol has its advocates and comes close to picrotoxin in life-saving capacity and, somewhat like picrotoxin, appears to stimulate much the same centers vitally concerned in profound barbiturate depression. Strychnine, on account of its dominant site of action on the cord and little action on medulla or midbrain, could not be expected to be of much value. Experience has supported this surmise. While strychnine rather effectively antagonizes the depression of the cord produced by barbiturates, when employed to antagonize more vital centers as are concerned with survival, such as the medulla and midbrain, it has little to recommend it. It may be stated that if life is not jeopardized through depression, either coramine, caffeine or strychnine may effect arousal.

The converse situation is rather different. Convulsions produced by

[3] Two cubic centimeters of a solution containing 12.5 per cent barbital soluble and 0.75 per cent pentothal per kilogram body weight.

most convulsants are strikingly controlled by appropriate barbiturates. Strychnine, cocaine, procaine, metrazol, picrotoxin, thujone, insulin in excess, etc., are all effectively controllable within certain ranges of dosages by symptomatic administration of barbiturates.

There are other examples of antagonisms. Meek and Seevers demonstrated that barbital is quite protective against cardiac irregularities caused by ephedrine in dogs. They believe the action of barbital to be due partially to a vagal depression and partially to depression of automatic centers. Hoff and Nahum (66) found amytal effective in preventing cardiac irregularities due to calcium. Acetyl salicylic acid appears to antagonize to a considerable extent the central depression produced by barbital or phenobarbital (Gilman and Barbour) (45). The mechanisms involved in this instance are quite obscure.

By and large, barbiturates appear to be surprisingly effective in controlling convulsions of most organs in that they appear to be selectively depressant on centers or pathways involved in the convulsive processes with, at the same time, less direct action on centers controlling respiration and vasomotor activity. This is illustrated particularly well in the instance of cocaine convulsions. Cocaine certainly poisons medullary as well as other centers, yet the immediate local effect of cocaine on the respiratory and vasomotor mechanisms can be tolerated providing the animals be functionally or anatomically decerebrated. The barbiturates appear to functionally decerebrate without at the same time seriously impairing vital medullary centers (172). The anesthetics, on the other hand, appear equally capable of blocking out convulsive processes, yet they seem to be so much less specific in action that the vital centers are apt to be simultaneously depressed even to the stage of paralysis. Thus, the amount or concentration of ether or chloroform necessary to block out convulsive processes also seriously and dangerously depresses the medulla. In strychnine poisoning the situation is somewhat different in that this drug acts chiefly on the cord with but little evidence of damage to the medulla; hence a general anesthetic would appear to be appropriate in that cord depression or paralysis occurs before a significant depression of medullary centers develops. By the same reasoning, barbiturates, which act chiefly and dominantly on the brain, would not appear to be particularly appropriate even though the cord does partake of the depressive action of the barbiturates as demonstrated and measured by Porter and Allamon.

Classification. Although Vogt (178) spoke of veronal as long in

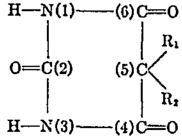
duration and slow in onset of action, phanodorn and amytal with a short period of onset and quick recovery, nostal and pernoston with quickest recovery, Fitch and Tatum (40) were the first to recognize the practicability of using duration of action as a basis of classification. Although there are minor differences in quality of action in different depressant members of the very large series, they are, as a whole, strikingly alike except for duration of effects. These differences in duration are most exactly determined when they are administered intravenously in order to eliminate the factor of differences in rate of absorption, as occurs when they are administered orally. Werner, Pratt and Tatum (184) have emphasized this factor as especially prominent in rabbits. A drug listed as a short-acting agent may behave in these animals as long-acting owing to a large dose, a prolonged period of absorption, and a relatively rapid rate of destruction after absorption. Here the rate of destruction approximates the rate of absorption, thus preventing great accumulation in body tissues. Fulness of the stomach and gastric motility also appear to be important modifying factors. Capacity of the liver and other organs to destroy these drugs must also be considered. Together, these authors proposed four groups into which the depressant barbiturates may fall, viz., long, intermediate, short and ultrashort-acting drugs. Barbital and phenobarbital are representatives of long actors, neonal and dial as intermediate, amytal and nembutal as short, and finally, evipal and pentothal as ultrashort-acting barbiturates. While there are members occupying a borderline status and hence not readily placed, the idea as a whole permits the rational selection of one or other of the ever-increasing series, according to experimental or clinical needs.

Some writers have written of some of the members as having a quick action, i.e., short induction period, whereas others, notably barbital and phenobarbital, as having a long induction period. It so happens that, with the exception of barbital and phenobarbital, practically all of them have a short induction period and hence this basis of classification scarcely suffices to be of significant and practical value.

The classification of duration of action is significantly applicable only to the depressant members of the series. It is well known that there are convulsant barbiturates, as described by Dox and Hjort (30), Shonle and Waldo (161), and Knoefel (84), but these, as yet, have not been found to possess any practically useful properties.

In the experimental field it is extremely difficult to demonstrate

Plan of classification



Duration	Group	Compound		(5) R_1	(5) R_2	(1)	(2)
increase ↑	LONG	Phenobarbital (Luminal)	USP	ethyl	phenyl		
		Barbital (Medinal) (Veronal)	USP	ethyl	ethyl		
DURATION	INTERMEDIATE	Neonal	NNR	ethyl	n-butyl		
		Dial	NNR	allyl	allyl		
		Ipral	NNR	ethyl	isopropyl		
		Alurate	NNR	allyl	isopropyl		
		Nostal	NNR	bromallyl	isopropyl		
		Amytal	NNR	ethyl	isoamyl		
		Sandoptal	NNR	allyl	isobutyl		
	SHORT	Pentobarbital (Nembutal)	NNR	ethyl	l-methyl butyl		
		Phanodorn	NNR	ethyl	cyclohexenyl		
decrease ↓		Ortal	NNR	ethyl	n-hexyl		
	ULTRA SHORT	Evipal		methyl	cyclohexenyl	methyl	
		Pentothal		ethyl	l-methyl butyl		thio

hypnosis or sleep in laboratory animals by use of hypnotic drugs, since the various activities within the laboratory tend to keep such animals awake. Consequently, experimentalists are forced to depend upon symptoms such as incoördination and various stages of anesthesia rather than hypnosis or sleep. Hondelink (69) proposed the use of perching species of birds, finches, canaries, etc., as a means of differentiating hypnotic from anesthetic effects of drugs. This author claims that a bird at the hypnotic level of depression will remain quiet on the perch in a constantly lighted room, whereas if depression reaches the anesthetic levels the bird can no longer remain on the perch but falls to the floor of the cage. These birds may serve as suitable test objects for a comparative measure of true hypnotic potency of drugs.

Richter (146) measured depression effected by hypnotics by means of determinations of electrical skin resistance. He finds that skin resistance is markedly raised over the normal, even over the resistance of normal sleep. Abnormal resistance persists well beyond evident sleepiness. Richter ascribes this phenomenon to sweat gland activity and its central control through centers residing primarily in the hypothalamus. A change in the rate of blood flow is undoubtedly basic to this phenomenon and would appear to be a simpler explanation of the condition described by Richter.

Significance of structure. The relationship of structure to function is now such that certain generalities can be made.

1. It appears established that to be effective both hydrogen atoms on position *5* must be replaced by an alkyl or aryl group.
2. Increasing the length of one or both of the alkyl chains up to 5 or 6 carbons increases the potency, measured by dosage, and diminishes the duration of action.
3. Branched chains are apt to be shorter in action than straight chains.
4. Unsaturated short chains are more active than saturated short chains.
5. Alkyl groups on *1* or *3* lead to a shortening of period of action and tend to produce stimulation more or less in proportion to the length of the chain. Alkyl groups on both *1* and *3* are apt to produce convulsant action (30, 161, 84).
6. Replacement of oxygen on *2* by sulfur increases instability and shortens the period of action (58, 170).

Degradation products produced *in vivo* are as yet unidentified, except in active N-methyl compounds, in which case Butler and Bush (16) report that a considerable amount (40 per cent) of the demethylated analogues are formed.

In the instance of the thio-analogues, pentothal and thioethamyl, it is known that long continued intravenous injection, or repeated intravenous injections, lead to a progressive prolongation of depression (144). This is suggestive of cumulation but such is not the case, according to Kozelka and Hine (99) who have demonstrated that thiobarbiturates exist in the tissues of the dog for only a very short time (e.g., one hour), after which neither the thiobarbiturate nor its oxygen analogue can be found. This is true even at a time, in prolonged administrations, when the animal is still in profound depression, in which case positive identification of the oxygen analogue, if present, could be made without

difficulty. Obviously, then, the long continued use of a thiobarbiturate appears to lead to the accumulation of some unidentified depressant degradation product.

Toxicology. The etiology of poisoning is for the most part self-medication. In many cases, perhaps the greater proportion, the poisoning is intended suicide; others develop a psychic dependence, and hence take the drugs in too large dosages, long after need has ceased, and develop chronic poisoning; finally, there is that peculiar condition spoken of as "automatism" by Richards (145). In this condition the individual, having become accustomed to the use of the drugs, may at some time fail to have deep sleep develop. In this twilight zone, an individual does not exert normal inhibition and reason, and hence mechanically takes all the remaining tablets or capsules. In these cases the coroner's jury pronounces the cause of death as suicide, and so it is, but it should be recognized scientifically as unintentional or accidental suicide. The recognition of this possibility by the medical and pharmaceutical professions might well effect a reduction in the number of accidents of this nature.

Diagnosis of poisoning depends upon history, circumstantial evidence, and finally on chemical analysis of excreta, gastric contents, or tissues and body fluids. There have been developed recently methods of analysis which have given reliable results. The older tests, e.g., Millon's reagent, were non-specific; cobalt color tests are fairly specific qualitatively, if properly used, but rather unreliable for quantitative purposes. Kozelka and Tatum (96) have called attention to the fact that the cobalt color test is not strictly proportional to the amount of barbiturate tested but depends upon at least two variables; hence for unknown amounts of barbiturates, quantification by this method leaves much to be desired in an analytical way. Kozelka et al. (98) have reported a method for quantitative isolation of barbiturates in pure form such as can be determined gravimetrically and identified by melting point determinations. This method is less sensitive for small quantities than the cobalt color test but, in cases of medicolegal character, quantities below the sensitivity range of the gravimetric method are not apt to be of serious or significant toxicological importance. The distribution of barbiturates in tissues has been reported by Koppanyi (95) and by Kozelka (98) and their associates.

The spinal barrier is rather unexpectedly high since barbiturates pass into the spinal fluid very slowly and in very small quantities (41, 97). On the other hand, Purves-Stewart and Willcox (136) maintain that

large quantities are to be found in the spinal fluid of patients poisoned by the use of barbiturates, but they do not reveal the methods of analysis employed. In one of their reports the amount of a barbiturate recovered in an aliquot of spinal fluid examined by their analyst was such that by simple calculation one could prove that most of the barbiturate ingested must have been uniquely collected in the cerebrospinal fluid!

Pathological changes in tissues. Ravn (140) reported a series of studies on the urobilinogen and bile acid tests on some 100 cases in a psychiatric hospital. One-half the cases were given barbital over a period of one to five years. Controls received no barbiturates. Of those receiving barbiturates 64 per cent developed positive tests whereas in the control series but 32 per cent were positive. Thus, the incidence appeared to be higher in the treated series.

Birch (6) reported a case of jaundice due to phenobarbital. Since typical skin lesions appeared, the conclusion was natural that the liver condition was likewise due to susceptibility to phenobarbital. Vaizey (176) reported a case of toxic jaundice due to pentothal. Many writers have reported dermatitis as a common reaction to phenobarbital, while Jontofsohn (75) described in considerable detail, barbituric acid allergy. Maillard and Thomazi (107) reported a case of myalgia and arthralgia as rather clearly due to barbital or luminal. Landor and Salleh (100) saw an instance of widespread paralysis following the use of evipal. Feldweg (38) saw two cases of sudden respiratory paralysis in asthmatics after evipal, which led him to proscribe this hypnotic in asthmatic cases. Mussio-Fournier, Austt and Arribeltz (123) observed a case developing the parkinsonian syndrome and mental disturbances after chronic use of barbital. This observation is affirmed by the Ziskinds (187). These authors state quite positively that phenobarbital is contraindicated in parkinsonism.

Intoxication. Holck and Cannon (68) confirmed the observations of Fitch and Tatum on delayed death of rabbits after nostal and observed in connection therewith the occurrence of parenchymatous damage (fatty changes) particularly in the liver. Kennedy (80) saw parenchymatous degeneration occurring in mice subjected to chronic poisoning by evipal. Seevers and Tatum (157) reported a study of chronic barbital intoxication in dogs covering a period of over three years. Morphological changes were studied for these authors by Dr. Roy Grinker. He described well-marked morphological changes in the brains of these animals, which changes may well be basic to deportmental characteristics. In the dogs there was definite and clear-cut

evidence of a partial tolerance as well as changes in deportment. These deportmental changes and partial tolerance signs were entirely confirmed by Oettel and Krautwald (129). Fitch (39) reported partial tolerance development in rabbits toward a number of different barbiturates. Observations similar to those made by Fitch have been made by a number of investigators (19, 20, 21, 36, 114, 119, 125, 163, 174), whereas only Swanson, Weaver and Chen, in their study of amytal, denied tolerance development (169). Since Fitch, as well as others, found amytal to behave essentially like other destructible barbiturates, the evidence is conclusive that while rate of development and degree of tolerance may vary somewhat in different animals for different barbiturates, they (including amytal) do produce a demonstrable shortening of depression period on repetition of dosage. All of the work reported, except that by Seevers and Tatum and by Oettel and Krautwald, has been too hurriedly completed, i.e., not continued over a sufficiently long period of time to develop real chronic symptoms; hence relatively negative results over a short period are without weight in comparison to positive results obtained after a long period. One could well take the view that negative results would have to endure throughout the entire life of an animal before one could say that chronic poisoning does not occur. Certainly, a few weeks up to half a year would be significant only if positive results were obtained. It is perfectly well known that *in the clinical field, chronic poisoning by certain substances may appear* even after a period of twenty years.

Habituation. Repeated and continued use of barbiturates induces a condition of habituation or psychic dependence but apparently not a true addiction as exemplified by use of morphine or heroin. (See definitions by reviewer (173).)

Mental disturbances occur during the period of drug-taking and obviously are due to the positive action of the drugs rather than to the cessation of drug-taking or abstinence phenomena. Schmidt (154), on the other hand, describes what he considers to be withdrawal symptoms in his series of eleven human cases who had been taking phenobarbital for six or more years and appeared, therefore, to fit into the category of true addiction. These symptoms were well described by Mussio-Fournier et al. More recently, Weiss (182) and Robinson (149), among others, have discussed this problem. These writers also stress the possibility of irreparable damage to brain and other tissue cells resulting from abuse of these drugs, a condition in man more or less analogous to that described for the dog by Seevers and Tatum.

Treatment of acute poisoning. On the basis of experimental work on the treatment of profound poisoning of laboratory animals, as indicated previously in this review, effective analeptics appear to be limited to two agents, viz., metrazol and picrotoxin. In non-serious poisonings, caffeine, coramine and strychnine appear to be effective in causing arousal but do not raise the lethal dosage of barbiturates significantly. Owing to the uncertainty of dosage a human subject may have taken and his susceptibility thereto, a number of writers have extolled the efficacy of these experimentally weak analeptics. Doubtless these patients would have recovered without the use of these agents if we are to learn from animal experimentation. How much this may be true also for metrazol or picrotoxin cannot be definitely stated, but from such clinical reports as are now available, viz., Bleckwenn and Masten (10), Kohn, Platt and Saltman (87), Kline, Bigg and Whitney (83), Koppanyi and associates (95), and Rovenstine (152), it is clearly apparent that these clinical investigators are convinced that picrotoxin is the most effective and sustaining analeptic so far known for clinical barbiturate poisoning. The argument may be advanced that these cases might also have recovered without the use of powerful analeptics, but owing to uncertainty of exact predictability in any kind of therapy for any disease, a similar logic could be advanced against any therapeutic agent whatsoever. It is safe, therefore, to anticipate that those who are familiar with the evident potency of picrotoxin, properly administered, would elect to use it in serious cases of barbiturate poisoning since a small dosage of a potent drug will work in mild cases, and larger doses may work in serious cases, whereas weak analeptics will likely fail except in instances of minor and non-serious poisoning.

Most writers on this subject stress the importance of simultaneous use of other expedients such as oxygen, gastric lavage, and any other procedures as may be indicated. Cerebral edema requires appropriate attention.

Clinical indications. 1. *Hypnotic.* From what has been presented previously, hypnotic drugs may be of real value in establishing sleep in nervous insomnia. Since these drugs are not analgesic except in overwhelming dosages, pain as a cause of insomnia requires other therapy. The choice of hypnotic must depend upon circumstances (Weiss) such as the possibility that if sleep once is started it will continue more or less normally. For this purpose a short-acting drug would obviously be desirable. On the other hand, if a more or less continuous depression is required in order to effect sleep for a normal period, a longer-acting

drug is required. As a general rule, the short-acting barbiturates are freer from after-effects, i.e., mild depression, than long-acting drugs. If a more or less continuous depression is required through both day and night, a drug such as phenobarbital in repeated small doses very often gives satisfactory results. This is what is accomplished in epilepsy and also in hyperirritability of elderly people. In treatment of pertussis and seasickness, the short-acting barbiturates administered in small doses, symptomatically, appear to be logical and frequently satisfactory. In these instances the drugs are used in subhypnotic dosages.

2. *Pre-anesthetic depressant.* Many surgeons and anesthetists have found that the use of appropriate barbiturates may be satisfactorily employed to quiet a patient prior to anesthesia induction and thereby diminishing "psychic trauma." Many obstetricians are employing these drugs in the first stage of labor where it may not be desirable to too completely obtund labor pains. When used carefully in this way clinicians rarely see any evidence of fetal or infant depression in spite of the fact that experimentally these compounds can be demonstrated in fetal tissues, hence having passed through the placental barrier.

3. *Anesthetic.* The consensus of opinion of most students of anesthesia is that the barbiturates, with two exceptions, do not meet the requirements of anesthetic agents, particularly in regard to analgesia and to possibility of moment by moment control. The two exceptions that may be said to meet one of these requirements fairly closely are evipal and pentothal. These are ultrashort-acting barbiturates and if a "moment" be thought of as a period of five to fifteen minutes, then these agents would qualify in this particular.

4. *Anticonvulsant.* Against convulsions of cerebral origin, barbiturates have been found to be of particularly specific efficiency, as for example against cocaine, thujone, metrazol and picrotoxin, and less specific and satisfactory against cord convulsants such as strychnine. Nonspecific convulsions such as are met with in eclampsia also yield satisfactorily. Since eclampsia is either due to or accompanied by and contributed to by degenerative liver changes, parenchymatous poisons such as chloroform or chloral hydrate, as advocated by Strogonoff, would appear illogical providing there is available an equally effective agent which in itself is not a liver poison. The barbiturates appear to be the best answer available today. American obstetricians are more and more generally employing one or other of the short-acting barbiturates. Even here a certain caution must be raised since in conditions of liver deficiency, destruction of short-acting barbiturates will take place less

readily than in normals, thus leading to a longer action than otherwise would be expected. Since convulsions in themselves are exhausting, it is imperative to keep them from appearing. Other measures are still to be used since convulsions are clearly due to something other than lack of "hypnotics" in the system. In all instances the control of convulsions should be by careful symptomatic administration of an antagonistic drug. The severity of convulsions scarcely suffices to indicate the amount of drug to be given. The practical difficulty of intravenous injection of a hypnotic drug to a person in convulsions may require the use of a volatile anesthetic. This is particularly true of strychnine convulsions.

5. *Neuropsychiatry.* Bleckwenn (7, 9) and Lorenz (102, 103) and their associates first led the way to the psychic analysis of schizophrenias such as dementia praecox. They used first amytal for this purpose but are quite impartial in that they recognize that any short-acting barbiturate will serve the same purpose. Their technique is to administer the barbiturate intravenously and slowly until the corneal reflex is abolished. The patient then is allowed to come out from the effect of the drug. As he comes out there will be a period of a few minutes during which time the physician can converse with the patient. As the effects of the drug diminish further the patient is apt to lapse back into the previously existing noncommunicative state. This would look as though the psychosis were a condition of excessive inhibition, which inhibition could temporarily be removed by a depressant drug. Psychiatrists, no doubt, would express it otherwise, but to a layman in this field the expression *removal of inhibition* conveys the general idea in common language. This view is so expressed by Herman (61). An excellent review of the literature on the use of barbiturates in neuropsychiatric conditions was made by Wagner in 1933 (180).

The use of barbiturates as "truth serum" has appealed to the popular mind although there is nothing either particularly new or certain in its use. It has been known since antiquity that alcoholic intoxication releases the subject from inhibitions and ordinary control, and it appears that any depressant may simulate this action providing the "stages" of anesthesia are not too rapid in their transition. The barbiturates are rather less apt to produce delirium than is scopolamine previously used for this purpose, and hence probably slightly more reliable in action.

The use of these hypnotic drugs for maintenance of prolonged depression in the treatment of psychic disturbances is an old procedure and is still advocated by a number of neuropsychiatrists. Lutz (106), Palmer

and Paine (132), Menzies (116), Jenny (72), and Wilson and Gillman (185) report favorable results, particularly in patients whose abnormalities are of not too long duration. The current practice is to provide the patient with a prolonged and enforced mental rest whereby the habit of *psychotic activity may be broken through inactivity.*

The use of phenobarbital in treatment of epilepsy appears to depend upon a long continued but mild cortical depression and is still, generally speaking, the drug of choice, though "dilantin," a non-hypnotic drug, has effected a peculiar and unanticipated controlling action (117, 118). It is thus possible that a better understanding of idiopathic epilepsy may be obtained through this interesting, though not entirely safe, hydantoin derivative.

Posology

SPECIES	DRUG	ADMINISTRATION	HYPNOTIC DOSE	ANESTHETIC DOSE	M.L.D.	AUTHORITY
			mgm./kgm.	*mgm./kgm.*	*mgm./kgm.*	
Chimpanzee	Amytal	i.p.		65		Fulton and Keller (43)
	Dial	i.p.		55		Fulton and Keller (43)
	Pentobarbital	i.p.		40		Fulton and Keller (43)
	Pentobarbital (Nembutal)	rectal		25–30		Elder (32)
Rhesus	Dial	i.p.		40		Fulton, Liddell and Rioch (42)
Horse	Pentobarbital	i.v. or i.p.		9.5		Abbott Laboratories
Cattle	Pentobarbital	i.v. or i.p.		9.5		Abbott Laboratories
Sheep	Pentobarbital	i.v. or i.p.		28		Abbott Laboratories
Swine	Pentobarbital	i.v. or i.p.		28		Abbott Laboratories
Dogs	Amytal	i.v.		58		Holck and Kanan (67)
	Amytal	i.p.		50–70		Mulinos (120)
	Amytal	i.p.		34–44		Hafkesbring and MacCalmont (59)
	Amytal	i.v.		45–60		Page and Coryllos (131)
	Amytal	i.p.		50		Rakieten et al. (139)
	Barbital	oral		250		Tatum and Parsons (171)
	Barbital	i.v.		250		Unpublished data (Tatum)
	Barbital	i.p.		180		Hafkesbring and MacCalmont (59)
	Dial	i.p.		50		Rakieten et al. (139)
	Dial	i.p.		50		Fulton, Liddell and Rioch (42)
	Nostal	oral		20–35		Boedecker and Ludwig (11)
	Ortal	i.v.		40		Gruber and Brundage (54)
	Pentobarbital	i.v. or i.p.		28		Abbott Laboratories
	Pentobarbital	i.p.		45–55		Mulinos (120)
	Pentobarbital	i.p.		22–25		Hafkesbring and MacCalmont (59)
	Pentobarbital	i.p.		30		Rakieten et al. (139)
	Pentobarbital	i.p.		22–45		Bazett and Erb (2)
	Pentothal	i.v.		15–20		Pratt et al. (135)
	Pernoston	i.p.		40–60		Mulinos (120)
	Phenobarbital	i.p.		20		Rakieten et al. (139)
	Seconal	i.v.		25		Swanson (168)
Cats	Amytal	oral			100	Eddy (31)
	Amytal	i.p.		60–80		Mulinos (120)
	Amytal	i.p.		24–25		Hafkesbring and MacCalmont (59)
	Barbital	oral			280	Eddy (31)
	Dial	i.p.		50		Fulton, Liddell and Rioch (42)
	Ipral	oral			140	Eddy (31)
	Neonal	oral			84	Eddy (31)
	Pentobarbital	i.p.		18		Hafkesbring and MacCalmont (59)
	Pentobarbital	i.p.		22–45		Bazett and Erb (2)
	Pentobarbital	i.v. or i.p.		28		Abbott Laboratories
	Pentobarbital	i.p.		45–55		Mulinos (120)
	Phanodorn	oral			120	Eddy (31)

Posology—Concluded

SPECIES	DRUG	ADMINISTRATION	HYPNOTIC DOSE	ANESTHETIC DOSE	M.L.D.	AUTHORITY
			mgm./kgm.	*mgm./kgm.*	*mgm./kgm.*	
Rabbits	Amytal	i.v.		45	75	Holck and Kanan (67)
	Amytal	i.p.		55–60	90	Fitch and Tatum (40)
	Barbital	i.p.			225	Fitch and Tatum (40)
	Evipal	i.p.		70		Maloney and Hertz (109)
	Ipral	i.p.			110	Fitch and Tatum (40)
	Narconumal	i.v.		20	50	Demole (24)
	Neonal	i.p.			115	Fitch and Tatum (40)
	Nostal	i.p.			120	Fitch and Tatum (40)
	Pentobarbital	i.p.		30-40		Mulinos (120)
	Pentobarbital	i.p.		40	65	Fitch and Tatum (40)
	Pentothal	i.v.		20	35	Pratt et al. (135)
	Pentothal	oral		360	600	Pratt et al. (135)
	Pernoston	i.p.		90-100		Mulinos (120)
	Pernoston	subcut.		50	160	McCarthy (124)
	Pernoston	i.p.			75	Fitch and Tatum (40)
	Phanodorn	i.p.			130	Fitch and Tatum (40)
	Phenobarbital	i.p.			150	Fitch and Tatum (40)
Guinea pigs	Evipal	i.p.		100		Kennedy (80)
	Pentobarbital	i.p.			45-60	Carmichael and Posey (20)
Rats	Amytal	subcut.		100-250		Nicholas and Barron (125)
	Amytal	i.p.			115	Fitch and Tatum (40)
	Barbital	i.p.			300	Fitch and Tatum (40)
	Evipal	i.p.		100		Maloney and Hertz (109)
	Neonal	i.p.			135	Fitch and Tatum (40) Nielsen et al. (126)
	Ortal	i.p.		100-110		Gruber and Brundage (54)
	Pentobarbital	i.p.			75	Fitch and Tatum (40)
	Pentothal	i.p.		80		Pratt et al. (135)
	Phenobarbital	i.p.			155	Fitch and Tatum (40)
	Seconal	i.p.		40		Swanson (168)
Mice	Evipal	i.p.		95	190	Kennedy (80)
Fowls	Barbital	i.v.	150		225	Koppanyi et al. (90)
	Dial	i.v.	60			Koppanyi et al. (90)
	Neonal	i.v.	60			Koppanyi et al. (90)
	Pentobarbital	i.v.	25			Koppanyi et al. (90)
	Pernoston	i.v.	20			Koppanyi et al. (90)
	Phenobarbital	i.v.	100			Koppanyi et al. (90)
Chicks	Pentothal	i.v.		20		Klein, B. E. (personal communication)
Pigeons	Barbital	i.m.		30		Winiwarter (186)
	Evipal	i.m.		4		Winiwarter (186)
	Pernoston	i.p.		30-40		Mulinos (120)
	Phenobarbital	i.m.		12		Winiwarter (186)
Finches	Barbital	i.m.		125		Houdelink (69)
Turtles	Barbital	i.v.		200-300		Koppanyi et al. (90)
Frogs	Evipal	i.p.			30-80	Kennedy (80)
	Pernoston	subcut.		60	150	McCarthy (124)
Fish	Amytal	i.p.		40-54	60	Keys and Wells (81)

REFERENCES

(1) ANDERSON, CHEN AND LEAKE. J. Pharmacol. and Exper. Therap. **40**: 215, 1930.
(2) BAZETT AND ERB. Ibid. **49**: 352, 1933.
(3) BEHNECKE. Arch. exper. Path. u. Pharmakol. **163**: 594, 1932.
(4) BERUTTI. Ginecologia Torino **2**: 1237, 1936; C. A. **32**: 9283, 1938.
(5) BETLACH. J. Pharmacol. and Exper. Therap. **61**: 329, 1937.
(6) BIRCH. Lancet **1**: 478, 1936.
(7) BLECKWENN. Arch. Neurol. and Psych. **24**: 365, 1930.
(8) BLECKWENN. J. A. M. A. **95**: 1168, 1930.
(9) BLECKWENN. Wisconsin Med. J. **29**: 693, 1930.
(10) BLECKWENN AND MASTEN. J. A. M. A. **111**: 504, 1938.
(11) BOEDECKER AND LUDWIG. Klin. Wchnschr. **3**: 2053, 1924.
(12) BOUCKAERT AND HEYMANS. J. Physiol. (Proc.) **90**: 15, 1937.
(13) BOYD. Quart. J. Pharm. Pharmacol. **10**: 659, 1937.
(14) BURSTEIN AND ROVENSTINE. J. Pharmacol. and Exper. Therap. **63**: 42, 1938.
(15) BURSTEIN. Proc. Soc. Exper. Biol. and Med. **40**: 122, 1939.
(16) BUTLER AND BUSH. J. Pharmacol. and Exper. Therap. (Proc.) **63**: 4, 1938.
(17) CALDERONE. Ibid. **55**: 24, 1935.
(18) CAMERON AND DE SARAM. J. Path. and Bact. **48**: 49, 1939.
(19) CARMICHAEL AND POSEY. Proc. Soc. Exper. Biol. and Med. **30**: 1329, 1933.
(20) CARMICHAEL AND POSEY. J. Pharmacol. and Exper. Therap. (Proc.) **57**: 116, 1936.
(21) CARMICHAEL AND POSEY. Anes. and Analg. **16**: 156, 1937.
(22) DAMESHEK, MYERSON AND LOMAN. Am. J. Psychiat. **91**: 113, 1934.
(23) DAWSON AND PORTER. Proc. Soc. Exper. Biol. and Med. **31**: 1048, 1934.
(24) DEMOLE. Compt. rend. Ier Congres International de l'Union Therapeutique, Berne, May 19–22, 1937.
(25) DE NITO. Boll. soc. ital. biol. sper. **12**: 240, 1937.
(26) DE WAELE. Arch. Internat. d. Physiol. **25**: 83, 1925.
(27) DILLE. J. Pharmacol. and Exper. Therap. **52**: 129, 1934.
(28) DILLE AND AHLQUIST. Ibid. **61**: 385, 1937.
(29) DORFMAN AND BROOKS. Ibid. (Proc.) **57**: 119, 1936.
(30) DOX AND HJORT. Ibid. **31**: 455, 1927.
(31) EDDY. Ibid. **33**: 43, 1928.
(32) ELDER. Ibid. **60**: 347, 1937.
(33) ELMAN, WEINER AND COLE. Proc. Soc. Exper. Biol. and Med. **32**: 793, 1935.
(34) EMGE AND HOFFMAN. Anes. and Analg. **10**: 88, 1931.
(35) ESSEX, SEELEY, HIGGINS AND MANN. Proc. Soc. Exper. Biol. and Med. **35**: 154, 1936.
(36) ETTINGER. J. Pharmacol. and Exper. Therap. **63**: 82, 1938.
(37) FABRE. Compt. rend. Soc. de Biol. **113**: 1380, 1933.
(38) FELDWEG. Münch. med. Wchnschr. **81**: 257, 1934.
(39) FITCH. J. Pharmacol. and Exper. Therap. (Proc.) **39**: 266, 1930.
(40) FITCH AND TATUM. Ibid. **44**: 325, 1932.
(41) FRETWURST AND VOSS. Klin. Wchnschr. **15**: 126, 1936.

(42) Fulton, Liddell and Rioch. J. Pharmacol. and Exper. Therap. 40: 423, 1930.
(43) Fulton and Keller. *Surg., Gynec. and Obst.* 54: 764, 1932.
(44) Garry. J. Pharmacol. and Exper. Therap. 39: 129, 1930.
(45) Gilman and Barbour. Proc. Soc. Exper. Biol. and Med. 32: 1634, 1935.
(46) Gouaux, Cordill and Eaton. J. Lab. and Clin. Med. 22: 704, 1937.
(47) Gruber and Baskett. J. Pharmacol. and Exper. Therap. 25: 219, 1925.
(48) Gruber and Roberts. Ibid. 27: 327, 1926.
(49) Gruber. Ibid. 30: 149, 1926.
(50) Gruber. Ibid. 33: 201, 1928.
(51) Gruber, Crawford, Greene and Drayer. Ibid. 42: 27, 1931.
(52) Gruber, Scholten, DeNote and Wilson. Ibid. 56: 341, 1936.
(53) Gruber. Ibid. 60: 143, 1937.
(54) Gruber and Brundage. Ibid. 60: 439, 1937.
(55) Gruber, Haury and Gruber. Ibid. 63: 193, 1938.
(56) Gruber, Gruber and Colosi. Ibid. 63: 215, 1938.
(57) Gruber, Haury and Gruber. Ibid. 63: 229, 1938.
(58) Gruhzit, Dox, Rowe and Dodd. Ibid. 60: 125, 1937.
(59) Hafkesbring and MacCalmont. Ibid. 64: 43, 1938.
(60) Halsey and Lacey. Proc. Soc. Exper. Biol. and Med. 30: 1429, 1933.
(61) Herman. Psychiat. Quart. 12: 738, 1938.
(62) Herwick. J. Pharmacol. and Exper. Therap. 39: 267, 1930.
(63) Herwick. *Ibid.* 42: 268, 1931.
(64) Hesse, Baumgart and Dickmann. Klin. Wchnschr. 11: 1665, 1932.
(65) Hirschfelder and Haury. Proc. Soc. Exper. Biol. and Med. 30: 1059, 1933.
(66) Hoff and Nahum. J. Pharmacol. and Exper. Therap. 60: 425, 1937.
(67) Holck and Kanan. J. Lab. and Clin. Med. 19: 1191, 1205, 1934.
(68) Holck and Cannon. J. Pharmacol. and Exper. Therap. 57: 289, 1936.
(69) Hondelink. Arch. f. exper. Path. u. Pharmakol. 163: 662, 1932.
(70) Horsley. Lancet 1: 141, 1937.
(71) Isenberger. Am. J. Surg. 9: 35, 1930.
(72) Jenny. Am. J. Psychiat. (supp.) 94: 184, 1938.
(73) Johnston. J. Pharmacol. and Exper. Therap. 57: 333, 1936.
(74) Johnston. J. Pharmacol. and Exper. Therap. 64: 330, 1938.
(75) Jontofsohn. Med. Klinik 29: 189, 1933.
(76) Jowett and Quastel. Biochem. J. 31: 565, 1937.
(77) Jowett. J. Physiol. 92: 322, 1938.
(78) Keeser, E. and I. Keeser. J. Pharmacol. and Exper. Therap. 53: 137, 1935.
(79) Keeser, E. Arch. f. exper. Path. u. Pharmakol. 186: 449, 1937.
(80) Kennedy. J. Pharmacol. and Exper. Therap. 50: 347, 1934.
(81) Keys and Wells. *Ibid.* 40: 115, 1930.
(82) Kleindorfer and Halsey. Ibid. 43: 449, 1931.
(83) Kline, Bigg and Whitney. J. A. M. A. 109: 328, 1937.
(84) Knoefel. J. Pharmacol. and Exper. Therap. (Proc.) 57: 130, 1936.
(85) Kohn and Lederer. J. Lab. and Clin. Med. 23: 717, 1938.
(86) Kohn and Grimes. J. Pharmacol. and Exper. Therap. (Proc.) 63: 18, 1938.

(87) Kohn, Platt and Saltman. J. A. M. A. **111**: 387, 1938.
(88) Kolb and Langworthy. J. Pharmacol. and Exper. Therap. **63**: 108, 1938.
(89) Koppanyi, Murphy and Krop. Arch. internat. de pharmacodyn. et de therapie **46**: 76, 1933.
(90) Koppanyi, Murphy and Gray. J. Pharmacol. and Exper. Therap. **52**: 78, 1934.
(91) Koppanyi and Krop. Ibid. **52**: 87, 1934.
(92) Koppanyi and Dille. Ibid. **54**: 84, 1935.
(93) Koppanyi, Linegar and Dille. Science **82**: 232, 1935.
(94) Koppanyi, Dille and Linegar. J. Pharmacol. and Exper. Therap. **58**: 119, 1936.
(95) Koppanyi, Linegar and Dille. Ibid. **58**: 199, 1936.
(96) Kozelka and H. J. Tatum. Ibid. **59**: 54, 1937.
(97) Kozelka and H. J. Tatum. Ibid. **59**: 63, 1937.
(98) Kozelka, H. J. Tatum and D. E. Nelson. Ibid. (Proc.) **63**: 19, 1938.
(99) Kozelka and Hine. Ibid. (Proc.) **66**: 20, 1939.
(100) Landor and Salleh. Brit. Med. J. **2**: 940, 1934.
(101) Lieb and Mulinos. Proc. Soc. Exper. Biol. and Med. **26**: 708, 1929.
(102) Lorenz. Psychiat. Quart. **4**: 95, 1930.
(103) Lorenz. Wisconsin Med. J. **31**: 245, 1932.
(104) Lundy and Osterberg. Proc. Staff Meetings of Mayo Clinic **4**: 386, 1929.
(105) Lundy. Anes. and Analg. **9**: 210, 1930.
(106) Lutz. Ztschr. f. d. ges. Neurol. u. Psychiat. **123**: 91, 1930.
(107) Maillard and Thomazi. Presse Med. **39**: 851, 1931.
(108) Maloney, Fitch and Tatum. J. Pharmacol. and Exper. Therap. **41**: 465, 1931.
(109) Maloney and Hertz. Ibid. **54**: 77, 1935.
(110) Marshall and Rosenfeld. Ibid. **57**: 437, 1936.
(111) Marshall, Walzl and LeMessurier. Ibid. **60**: 472, 1937.
(112) Marx. Ibid. **41**: 483, 1931.
(113) Masserman. Arch. Neurol. and Psychiat. **37**: 617, 1937.
(114) Masuda, Budde and Dille. J. Am. Pharmaceut. Assn. **27**: 830, 1938.
(115) Meek and Seevers. J. Pharmacol. and Exper. Therap. **51**: 287, 1934.
(116) Menzies. Lancet **2**: 559, 1937.
(117) Merritt, Putnam and Schwab. Arch. Neurol. and Psychiat. **39**: 1003, 1938.
(118) Merritt and Putnam. J. A. M. A. **111**: 1068, 1938.
(119) Moir. J. Pharmacol. and Exper. Therap. **59**: 68, 1937.
(120) Mulinos. Arch. internat. de pharmacodyn. et de therapie **47**: 111, 1934.
(121) Murphy and Koppanyi. Proc. Soc. Exper. Biol. and Med. **31**: 376, 1933.
(122) Murphy and Koppanyi. J. Pharmacol. and Exper. Therap. **52**: 70, 1934.
(123) Mussio-Fournier, Austt and Arribeltz. Bull. et Mem. de la Soc. Med. des Hopitaux de Paris **47**: 1748, 1931.
(124) McCarthy. Anes. and Analg. **9**: 231, 1930.
(125) Nicholas and Barron. J. Pharmacol. and Exper. Therap. **46**: 125, 1932.
(126) Nielsen, Higgins and Spruth. Ibid. **26**: 371, 1925.
(127) Nomura. Nagoya Igakkai Zasshi **44**: 911, 1936; C.A. **32**: 5919, 1938.
(128) Nowak. Compt. rend. Soc. de Biol. **116**: 642, 1934.

(129) Oettel and Krautwald. Klin. Wchnschr. **16**: 299, 1937.
(130) Ogden. Proc. Soc. Exper. Biol. and Med. **27**: 506, 1930.
(131) Page and Coryllos. J. Pharmacol. and Exper. Therap. **27**: *189*, 1926.
(132) Palmer and Paine. Am. J. Psychiat. **12**: 143, 1932.
(133) Porter and Allamon. J. Pharmacol. and Exper. Therap. **58**: 178, 1936.
(134) Pratt. Ibid. **48**: 285, 1933 (Proc.).
(135) Pratt, Tatum, Hathaway and Waters. Am. J. Surg. **31**: 464, 1936.
(136) Purves-Stewart and Willcox. Lancet **1**: 500, 1934.
(137) Quastel and Wheatley. Proc. Royal Soc. **112B**: 60, 1932.
(138) Quigley and Phelps. J. Pharmacol. and Exper. Therap. **50**: 420, 1934.
(139) Rakieten, Nahum, DuBois, Gildea and Himwich. Ibid. **50**: 328, 1934.
(140) Ravn. Hospitalstidende **76**: 1103, 1933.
(141) Redenz. Münch. med. Wchnschr. **83**: 261, 1936.
(142) Reese. Wisconsin Med. J. **32**: 530, 1933.
(143) Reynolds. Proc. Soc. Exper. Biol. and Med. **28**: 656, 1931.
(144) Reynolds and Veal. *Ibid.* **37**: 627, 1938.
(145) Richards. Brit. Med. J. **1**: 331, 1934.
(146) Richter. J. Pharmacol. and Exper. Therap. **42**: 471, 1931.
(147) Robbins, Baxter and Fitzhugh. Ibid. **63**: 32, 1938 (Proc.).
(148) Robinson, C. C. Ind. State Med. Assn. J. **28**: 662, 1935.
(149) Robinson, G. W., Jr. Missouri State Med. Assn. J. **34**: 374, 1937.
(150) Roth. Proc. Soc. Exper. Biol. and Med. **30**: 563, 1933.
(151) Roth. Arch. internat. de pharmacodyn et de therapie **51**: 170, 1935.
(152) Rovenstine. Am. J. Med. Sci. **196**: 46, 1938.
(153) Rowbotham. Lancet **1**: 439, 1931.
(154) Schmidt. Münch. med. Wchnschr. **85**: 1944, 1938.
(155) Schriever and Perschmann. Pflüger's Arch. **236**: 497, 1935.
(156) Schriever and Perschmann. Compt. rend. Soc. de Biol. **120**: 971, 1935.
(157) Seevers and Tatum. J. Pharmacol. and Exper. Therap. **42**: 217, 1931.
(158) Seevers. J. Lab. and Clin. Med. **19**: 202, 1933.
(159) Shapiro. J. Nervous and Mental Dis. **85**: 305, 1937.
(160) Shonle, Keltsch, Kempf and Swanson. J. Pharmacol. and Exper. Therap. **49**: 393, 1933.
(161) Shonle and Waldo. J. Am. Chem. Soc. **55**: 4649, 1933.
(162) Siebert and Thurston. Proc. Soc. Exper. Biol. and Med. **29**: 650, 1932.
(163) Stanton. J. Pharmacol. and Exper. Therap. **57**: 245, 1936.
(164) Stavraky. Ibid. **43**: 499, 1931.
(165) Stormont, Lampe and Barlow. Ibid. **39**: 165, 1930.
(166) Swanson and Shonle. Ibid. **41**: 289, 1931.
(167) Swanson and Shonle. J. Lab. and Clin. Med. **16**: 1056, 1931.
(168) Swanson. Proc. Soc. Exper. Biol. and Med. **32**: 1563, 1935.
(169) Swanson, Weaver and Chen. Am. J. Med. Sci. **193**: 246, 1937.
(170) Tabern and Volwiler. J. Am. Chem. Soc. **57**: 1961, 1935.
(171) Tatum and Parsons. J. Lab. and Clin. Med. **8**: 64, 1922.
(172) Tatum, Atkinson and Collins. J. Pharmacol. and Exper. Therap. **26**: 325, 1925; Arch. Int. Med. **38**: 405, 1926.
(173) Tatum and Seevers. Physiol. Rev. **11**: 107, 1931.
(174) Taylor and Lackay. Proc. Soc. Exper. Biol. and Med. **33**: 621, 1936.

(175) Tyson, Shrader and Perlman. J. Ped. **13**: 86, 1938.
(176) Vaizey. Brit. J. of Anes. **15**: 55, 1938.
(177) Vogeler and Kotzoglu. Chirurg (Berlin) **7**: 242, 1935; Internat. Surg. Digest **20**: 18, 1935.
(178) Vogt. Arch. f. exper. Path. u. Pharmakol. **152**: 341, 1930.
(179) Volpitto and Marangoni. J. Lab. and Clin. Med. **23**: 575, 1938.
(180) Wagner. J. A. M. A. **101**: 1787, 1933.
(181) Walton. J. Pharmacol. and Exper. Therap. **47**: 141, 1933.
(182) Weiss. J. A. M. A. **107**: 2104, 1936.
(183) Werner. J. Pharmacol. and Exper. Therap. (Proc.) **60**: 122, 1937.
(184) Werner, Pratt and Tatum. Ibid. **60**: 189, 1937.
(185) Wilson and Gillman. J. Mental Sci. **84**: 991, 1938.
(186) Winiwarter. Arch. f. exper. Path. u. Pharmakol. **185**: 95, 1937.
(187) Ziskind, E. and E. S. Ziskind. J. A. M. A. **109**: 20, 1937.

MUSCLE HEMOGLOBIN

G. A. MILLIKAN

Physiological Laboratory, Cambridge, England

About one-fourth of the body's hemoglobin is not able to circulate in the blood, being locked up in the muscle fibres. This large fraction of the body's pigment has received but scant attention at the hands of physiologists and biochemists until quite recently, and even today it is the subject of less than one hemoglobin paper in twenty. Yet muscle hemoglobin is an exceptionally interesting substance in the beautiful simplicity of its physico-chemical behavior, in its physiological rôle, and in the insight which it is able to give us into aerobic muscular activity. The reason for our apathy is not far to seek: the pigment could neither be studied conveniently *in situ* nor easily extracted in a purified form. At the same time blood hemoglobin's obvious *function* as an oxygen carrier, coupled with the tempting ease with which it can be obtained, made it an all too successful competitor as an object of research. Since the isolation and crystallization of muscle hemoglobin by Theorell in 1932 (40), our knowledge has rapidly increased, though there is still a host of problems concerning the formation, disappearance, and certain aspects of its behavior in the body which have not yet even been tackled.

Nomenclature. It is fortunate that so little confusion of meaning has arisen from the multiplicity of names and symbols which have been attached to this substance; as long as this remains the case the matter of nomenclature may be regarded as of minor importance. *Muscle hemoglobin* or *myohemoglobin* are satisfactory terms in that they imply nothing more than the "hemoglobin occurring in muscle," but both words are somewhat long and cumbersome to use. *Myoglobin,* proposed by Günther in 1921, when he became convinced that the muscle and blood pigments were different, has the advantage of being more compact. The present author slightly prefers the longer term because of his desire to retain one word which will embrace that large family of substances, no matter where they are found or what their molecular weights, which consist of conjugated proteins 1, capable of combining reversibly with oxygen and carbon monoxide; 2, capable of being

oxidized to a "met" compound; 3, possessing a characteristic type of spectrum, and 4, whose prosthetic group is reduced heme (from protoporphyrin). This family includes the blood pigments of all vertebrates, the invertebrate erythrocruorins of Svedberg, and the muscle pigment. It excludes chlorocruorin, which has a different heme group, the cytochromes and helicorubin, which do not combine reversibly with oxygen, hemerythrin, which does not contain heme, and the copper-containing hemocyanins.

Myochrome of Mörner is now never used. MacMunn's *myohaematin* is now known as cytochrome. MacMunn also saw muscle hemoglobin, but did not distinguish it from the blood pigment.

Notation. In this paper MHb = muscle hemoglobin; Hb = blood hemoglobin.

1. Occurrence. Muscle hemoglobin is generally found in large quantities in those muscles requiring slow, repetitive activity of considerable force. The highest concentrations are found in muscles whose complete cycle of activity takes the order of a second, and whose action must be maintained over long periods. Examples are the heart muscles of larger mammals, breast muscles of the larger flying birds such as the pigeon, and leg muscles of running animals such as the horse and the dog. Muscles which regularly contract more than two or three times a second usually have much cytochrome and but little or none of the hemoglobin. Examples of these are the wing muscles of flying insects (flies, wasps, bees, etc., which may contract with a frequency as high as 300 per second), and hearts of small birds and mammals such as canaries and mice. On the other hand, muscles which are called upon for repetitive activity of no very great power, such as the heart muscles of frogs, or for intense contractions with large intervals of rest between, such as the leg muscles of frogs, contain considerable quantities of cytochrome but no muscle hemoglobin.

This principle of distribution is not, however, quite universal. The occurrence of MHb among invertebrates is puzzling and sporadic. It is true as Ray Lankester pointed out (23) that some of the most active invertebrate muscles, such as the pharynx of the gasteropods *Limnaeus* and *Paludina*, do contain hemoglobin, even though the blood of these animals is blue or colourless; on the other hand, the great majority of invertebrate muscles, including most invertebrate hearts, contain no trace of this pigment though the more active ones are rich in cytochrome. All active muscles contain considerable amounts of cytochrome, whether they contain the muscle hemoglobin or not.

However, in a red muscle MHb may have a concentration 50 times that of the cytochrome, and the latter is thus easily masked. (For the relative occurrence of these two pigments see Keilin, 18.) A curious distribution of the pigment occurs in newborn infants, which contain considerable amounts of it in their diaphragms but none in their hearts, although the heart muscle is earlier put to work (Jonxis, 17).

Among mammals and birds we find a close correspondence between MHb concentration and the tissue's requirements for vigorous but slow repetitive activity; this principle may be followed not only from muscle to muscle within one species, but also from species to species, as well as from time to time in the life of a single individual. The physiological and chemical differences between red and white muscle have been thoroughly reviewed by D. M. Needham (28). Where both kinds occur in the same muscle, as for example the soleus (red) and gastrocnemius (pale) of the cat, it is found that the twitch of the red muscle is more prolonged, the tetanus is much more marked, its maximum is achieved at a much lower frequency of stimulus, and it can be maintained for a much longer time. To this point we will return when we consider the physiological rôle of the pigment.

Effect of age and activity. The same muscles may vary widely in redness from individual to individual and from time to time, depending upon the amount of use to which they are being put. Much the most complete and careful study of these variations was made by Whipple and his collaborators (47, 48, 49) on dogs.[1] Some of the results are given in table 1, from which it may be seen that whereas newborn pups have very pale muscles, they develop redness with increasing age and activity. If the motor nerve to a red muscle is cut, it is found that there is rapid depletion of the MHb (5, 48), although bleeding has but a slow and small effect. "The demands of the active muscle for its

[1] Estimates of the concentration of MHb made before about 1925 must be accepted with caution, because many of the earlier workers were unaware of the fact that much of the redness of muscle may be due to cytochrome; up to 1932 many writers regarded the muscle and blood pigments as being identical and not to be distinguished spectroscopically. Whipple was among these, but his work is reliable because he freed the muscle from Hb by simultaneous perfusion and bleeding, and because his method of extraction left cytochrome in the muscle mince. Watson (46) believes that Whipple's values were too high because of imbibition of fluid during extraction. If this objection is valid, it will not affect the significance of the *relative* results which are the important ones for our present purpose.

hemoglobin evidently are honoured as meticulously in the internal metabolism clearing house as are the demands for the blood-forming organs"—Whipple.

Abnormal appearance of MHb in urine. In certain pathological states, MHb appears in the urine. This condition has been studied very fully in the horse, where it is associated with extreme muscular stiffness (lumbago, myohemoglobinuria, Kreuzlähme) (Carlstrom, 7). The author knows of one human case in which the symptoms were

TABLE 1

MHb concentration in dogs in milligrams per 100 grams tissue

a. Age variations

AGE	MHb		Hb BLOOD
	Legs, back	Heart	
2 months	107	125	8,300
4 months	205	222	9,400
Adult	815	480	18,200
Old	680	480	15,700

b. Variations with exercise

	MHb		
	Diaphragm	Heart	Legs, back
Small quiet poodle	387	296	391
Average healthy dog	723	450	700
Hunting dog		770	1051

Data from Whipple (1926). All values are given in milligrams of pigment per 100 grams tissue or blood. Each figure of table 1a is the average for several dogs. The values will be of the same order for horses and cattle. For comparison, the concentration of cytochrome c in horse's heart (a rich source) is c. 40 mgm. per 100 grams (Keilin and Hartree, 20).

nearly identical with those of the equine disease; it was completely different from paroxysmal hemoglobinuria, though large quantities of pigment (MHb, not Hb) were excreted. In this case the patient's plasma potassium went up during exercise by about double the normal rise, suggesting that his muscle membranes were made unusually permeable during activity.

2. Properties. A comparative list of the principal known properties of MHb and Hb are given in table 2; a critical estimate of their probable reliability, the methods by which they were discovered, and

TABLE 2

Comparative properties of muscle hemoglobin and blood hemoglobin. For qualifications, see text

	MHb	Hb	REFERENCES
Some common properties			
1. Prosthetic group	Ferroheme		37, 32
2. Iron content	0.345%		40
3. Reversible combination with	O_2, CO		14, 43, 44
4. Reversible redox system Fe″, Fe‴			
Spectroscopic			
5. Position, alpha band, oxy-	581 mμ	577 mμ	27, 29, 21, 18, 31, 42
6. CO-O_2 span	3.1 mμ	4-6 mμ	29, 33, 42
Gas-binding			
7. Shape of dissociation curve	Hyperbolic	Sigmoid	14, 44, 15
8. Oxygen affinity	Very high	Moderate	
pO_2, ½ sat'n. pH 7.4, 37° horse	3 mm.	22 mm.	44
9. pH effect on diss'n curve	Small	Moderate or large	44, 15
10. Relative CO/O_2 affinity	20	500	33, 43
11. Kinetics, pH 7.4 20°C. horse:			
O_2 combination, millimol^{-1} sec^{-1}	19,000	4,000	24
O_2 dissociation, sec.$^{-1}$	37	40	
CO combination, millimol^{-1} sec^{-1}	300	130	
CO dissociation, sec.$^{-1}$	0.04	0.004	
Miscellaneous distinguishing properties			
12. Molecular weight	17,500	68,000	39, 41
13. Solubility, horse	Very great		26
14. Rate, alkali denaturation	Very slow		12
15. Precipitin reaction	Specific	Specific	13
16. Ease of oxidation to "met"	Very easy	Less easy	40
17. Iso-electric point, horse	6.99	6.78	40

some supplementary material are given in the section which follows. In general it may be said that all properties which have been found to be identical in *all* blood hemoglobins are also shared by the

muscle pigment. On the other hand, where the blood hemoglobins have small quantitative differences among themselves, the muscle hemoglobins have a range of values so distinct from that of their blood analogues as to justify the creation of a sub-class. This is most marked in properties (5, 6, 7, 8, 9), where half a dozen species have been studied comparatively.

The preparation of the pure crystalline material by Theorell was a great step forward, as it enabled us to be sure that the distinctive properties which had been observed in solutions containing MHb were not due to impurities or to the admixture of some of the blood pigment. Full details of his method of preparation, which has been repeated successfully by several other workers, are given in Theorell's paper (40). The essential steps are the water extraction of the pigment from minced muscle tissue (perfused horse's heart gives the best results), the precipitation of foreign proteins by basic lead acetate, removal of traces of Hb by dialysis against saturated ammonium sulfate solutions, and the final crystallization by further dialysis. Practically all of the properties listed in the table can be observed in non-purified extracts as well as in the crystallized preparation, but until this identity was proved, all our knowledge of the pigment was uncertain.

Table 2: authorities and supplementary material. (1, 2, 3 and 4) now command universal agreement. There is no need for further discussion.

(5, 6). A complete spectrophotometric study of crystalline horse muscle hemoglobin, including all its common derivatives, oxy, CO, reduced, "met" has been made by Theorell (42). It includes the spectral range from 250 mu to 650 mu.

The position of all the visible bands are displaced to the red, not merely the alpha band. This shift is due to the globin part of the molecule, as the hemes of MHb and Hb are identical. Furthermore, the recombination of heme from either source with globins, always gives the band position corresponding to the original source of the globin (33).

The 40 Ångstrom displacement to the red is a property of the pigment itself, and is not an artefact of extraction, since it is found *in situ* in the living tissue (29). Though there are some differences as to the exact location of the position of the alpha band maximum from species to species, and from one observer to another, all authors place it between 5800 and 5825Å (42, 31, 38, 29, 46, 21, 25), whereas the corresponding band in vertebrate Hb is never further to the red than 5780 Å.

The only invertebrate whose blood and muscle pigments have been *comparatively studied is Ascaris, for which Keilin found the* MHb alpha band position at 5802 A., and for the Hb band 5785 Å. The shift here is in the right direction but is smaller than in mammals.

The difference in band position gives us the most convenient methods of identifying and estimating MHb in the presence of Hb, as it is found that a very simple relation exists between this quantity, as determined with a Hartridge spectroscope, and the relative amounts of the two pigments in a mixture (Watson, 46, 25). A spectrophotometric procedure has also been given, using the relative absorption coefficients at 577 mu and at 580 mu as the criterion (38).

When oxygen is replaced by CO the bands shift by a much smaller amount for MHb than for Hb. The range for various Hb's is from 46 to 62 Å (1), that for MHb from 29 to 36 Å, depending on the species (33).

A slightly lower value for the absolute absorption coefficient has been found on several occasions for MHb than for Hb. These differences are probably not significant, being little greater than the errors of the experimental methods by which they were obtained.

(7, 8, 9) *Several gas-binding properties were worked out independently* and at nearly the same time by R. Hill (14, 15) in Cambridge, and H. Theorell (40–44) in Stockholm, using different preparations and different methods. Hill used dilute water extracts (0.05 per cent–0.1 per cent) from perfused hearts of dog, sheep, ox and goat; the gas equilibria were determined entirely in a Thunberg tube, using a simple and elegant method, the degree of saturation being determined spectroscopically. Theorell used his crystalline horse heart preparation, and measured the dissociation curves of concentrated solutions (2.5 per cent to 6.0 per cent) with the Van Slyke apparatus, using a technique in which the entire equilibrium process as well as the measurement of combined gas was carried out in the bulb of the machine. Hill always reached his equilibrium point from below, Theorell from above. In spite of all these differences in technique, the two workers were in essential agreement, finding always hyperbolic curves, high oxygen affinity, and small effect of pH on affinity, which is by itself strong presumption of the correctness of their findings. Theorell also investigated the effect of temperature on the dissociation curve, and found a Q_{10} of 2.58, corresponding to a heat of reaction of 17,500 calories, which is considerably less than the corresponding values for blood hemoglobin. (10) CO/O_2 affinity. Theorell at pH 6.95 obtained the following values: 20°C: 19.3; 37°C: 13.8; 15°C:(extrapolated) 28.1.

(11) Kinetic results were obtained by the streaming flow method of Hartridge and Roughton, using crystallized MHb from horse's heart. The "O_2 combination" reaction is the fastest one involving biological substances which has been directly measured.

(12) Theorell's original molecular weight determinations (41) gave a value fo 34,000 or mixtures of 34,000 and 68,000, but recent results obtained by the same ultracentrifugal method and in the same laboratory (39) give values of 17,200 and 17,500. All agree in finding average molecular weights less for MHb than for Hb, which explains the rapidity with which MHb when injected into the blood stream is passed through the kidneys into the urine. This had been first reported in 1902 by Camus and Pagniez (4) for rabbits and dogs; it has since been extended to horses (6, 7), and confirmed on the dog (13), whose threshold of renal excretion for MHb is about $\frac{1}{5}$ that for Hb.

(13) The solubility of COMHb at pH 6.6 and 25°C. was measured by Morgan (26), who found that $\log S = 8.00 - 0.94\left(\frac{\Gamma}{2}\right)$, where S = solubility in grams per litre, and $\frac{\Gamma}{2}$ is the ionic strength per litre. COMHb is quite soluble at pH 6.6 in buffer solutions up to 3M phosphate, where COHb has a solubility less than 1 part in 1,000,000. The great difference in solubility provides a method for quantitative separation of MHb from Hb.

(14) Haurowitz, using material from humans, found MHb very much more resistant to alkali denaturation than Hb. This difference has been confirmed by Brinkmann and Jonxis, who now use the principle as a method of isolating and purifying MHb.

(15) The precipitin reaction of MHb and Hb from the same animals is specific.

(16) All observers agree as to the much greater ease of oxidation of MHb. The author knows, however, of no published results on the redox potential of the system MHb-met MHb.

(17) There is not good agreement on absolute values, but several workers have reported that the iso-electric point of MHb is 0.1 to 0.2 pH unit more alkaline than that of Hb.

3. Physical chemistry. *MHb as a "primitive" respiratory pigment.* Muscle hemoglobin is our best example of a respiratory pigment whose behavior is simple. It possesses the hyperbolic dissociation curve about which blood physiologists have been dreaming for 40 years but have found, alas, so seldom in the laboratory. It contains only one heme

group per molecule, the molecular weight being 17,200. It has a nearly negligible hydrogen ion effect. Anthropomorphically speaking, muscle hemoglobin appears to be the first attempt of a creator who was primarily a physical chemist to produce a respiratory pigment, before he became aware of the biological desirability of putting a crimp in the dissociation curve, or of giving the pigment a Bohr effect.[2] It is an ideal substance on which to test those minimum mass action assumptions, which are the basis of all oxygen-binding theories, and on which the intermediate compound hypotheses of Adair and Pauling rest.

The simplest specification which we can imagine for a respiratory pigment is that its combination with oxygen should take place according to the equations:

$$MHb + O_2 \rightleftarrows MHbO_2$$

$$\frac{[MHbO_2]}{[MHb][O_2]} = K$$

in which the combination process is a bimolecular one, while the splitting-up is unimolecular. For such a substance the equilibrium dissociation curve can only be a rectangular hyperbola passing through the origin and asymptotic to the horizontal line of 100 per cent saturation. There can be any number of such curves, but they are all of the same shape and differ from each other only in the amount by which they are stretched out along the horizontal or oxygen tension axis. Each curve is defined by a single, unequivocal equilibrium constant, K, the expression for the curve being:

$$\text{degree of saturation} = \frac{Kp}{1 + Kp}$$

where p is the oxygen tension. Curves of this shape have been found to occur with MHb, no matter from what animals they have been obtained. They do not occur with Hb, except as a result of rather rough handling (see Forbes and Roughton, 8, and Roughton, 35, p. 456, for critical surveys), but they have been reproducibly found in the dialyzed hemocyanins of *Helix pomatia*, *Limulus*, and *Busycon* (30). These hemocyanins share with muscle hemoglobin the interesting property

[2] The sporadic occurrence of MHb among lower animals and its absence from many of those possessing blood hemoglobins of a mammalian type militate against any serious theory for the evolution of the hemoglobins along these lines. There is no evidence that MHb appears earlier in evolutionary development than Hb.

of having their dissociation curves but little affected by changes in pH. This presumably means that in all these pigments the point at which the oxygen is attached to the pigment molecule is sufficiently removed from the acidic and basic ionization groups to be little affected by them. That the two properties (hyperbolic curve and small Bohr effect) can be independent, however, is shown by the example of the worm *Urechis caupo*, whose blood hemoglobin possesses the sigmoid curve but no effect of pH.

Kinetic experiments. A very rigorous test of the mass action interpretation of the hyperbolic curve consists in measuring the rates at which combination and dissociation processes take place, and comparing the quotient of the two opposing velocity constants with the equilibrium constant. Agreement was in fact found within the limits of experimental error both for O_2 and CO (24). The principal onus for the high oxygen affinity of MHb was found to rest with the "on" reaction, which is over four times as fast as the corresponding one for Hb, half completion of the reaction being reached in 0.0004 sec. under the conditions of the experiment. The small effect of pH on the equilibrium dissociation curve was also reflected in the kinetic experiments, but here it was the "splitting-off" reaction which showed the difference: Hb alters its dissociation rate tenfold in a pH range of 2.5 units, MHb showed no change in rate under the same conditions.

Significance of the molecular weight. A hyperbolic curve, and *only* a hyperbolic curve, is to be predicted for a "baby" respiratory pigment, i.e., one which has only room for one molecule of oxygen per molecule of pigment. A curve of any other shape would seal the fate of all attempts to apply mass action considerations to hemoglobin right at the start. But MHb is the only baby pigment known, and the confirmation of the expected form of its curve is significant experimental evidence that the more complicated mass action theories (Hill, Adair, Pauling, etc.) are proceeding on correct basic assumptions.

The converse proposition does not hold good, for a hyperbolic curve does not necessitate a baby pigment. Such a curve may occur with a molecule containing any number of oxygen-binding groups provided simply that these different groups are independent of each other. This is the condition occurring in the giant hemocyanin molecules, which possess hyperbolic curves but have molecular weights of several millions.

Relation between CO/O_2 affinity and spectral "span." The small quantitative differences which characterize the various hemoglobins have defied all attempts to bring them into one orderly system, with the

exception of the one remarkable relation discovered by Anson, Barcroft, Mirsky and Oinuma (1) between gas-binding and spectroscopic properties. These authors found that the shift in position of the alpha band when CO replaced O_2 was larger for those hemoglobins which had a greater affinity for CO relative to O_2. For example, a dog whose partition constant, M,[3] was 550 had a "span"[4] of 61 Ångstroms, while a rabbit with a partition constant of 150 had a span of 48 Å. Furthermore, the data for all thirteen species examined showed that log M was directly proportional to the span. As one of the authors pointed out (2), however, the smallest span measured was 46 Å and the largest 62 Å, while the experimental error was large enough to make the straightness of the line somewhat questionable. Theorell's very careful data on MHb (43), and Roche's previous observations (33) enable us to test the phenomenon over a larger range, the span here being 32 Å and the partition constant 28.1. This point lies exactly on the original straight line, now extrapolated to over twice its previous length, and, in the view of the present writer, this confirmation establishes the relationship as a fundamental property of hemoglobin. It was pointed out in the original paper that the band displacement is proportional to the change in free energy of the system (since log K is a measure of this quantity), but unfortunately no more detailed explanation of this most interesting phenomenon in terms of physical chemistry has yet been made.

4. The rôle of muscle hemoglobin in the body. Muscle hemoglobin might serve the body in three capacities:[5] 1, as an agent in oxygen transport; 2, as an intracellular catalyst; 3, as an oxygen store.

1. Oxygen transport. Muscle hemoglobin could conceivably circulate inside the cell in the same manner as the blood circulates in the body, picking up oxygen at the periphery and giving it up to enzymes

[3] "Partition constant" = relative concentrations of O_2 and CO in equilibrium with Hb, which is 50 per cent in the form O_2Hb and 50 per cent in the form COHb =

$$M = \frac{[O_2][COHb]}{[CO][O_2Hb]}.$$

[4] "Span" = difference in position of the maximum of the alpha band between COHb and O_2Hb as measured e.g. with the Hartridge reversion spectroscope.

[5] The present treatment owes much to chapter V of Barcroft's *Architecture of Physiological Function* (1931). The considerable number of new experimental facts which have come to light in the last five years provide the only excuse for this supplement to that stimulating and readable chapter.

in the cell interior. Two circumstances make this rôle extremely unlikely: the lack of any apparent mechanism for circulation inside the muscle fibre, and the very high rate of diffusion over small distances, which would render such circulation of small value. Second, it might remain stationary itself but be of aid to the flow of oxygen molecules, by combining with them and releasing them in an oriented or activated state, so that they could be more readily handled by the cellular oxidases. But in this case MHb is by definition a catalyst. If, on the other hand, it combines with oxygen in a thermodynamically reversible manner, the released molecules of oxygen must be of precisely the same reactivity as those which have not yet been bound; if there were now a uniform flow of oxygen to the interior of the cell, the presence of the pigment in such an equilibrium state would be of no aid, for its removal would bring no change in the number of oxygen molecules made available each second for the use of the cell. True, in periods of fluctuating supply or demand, the pigment would help to even out this uneven flow, but in this case it would be acting simply as an oxygen store. The problem of oxygen transport has thus been transformed either into one of catalysis or into one of storage—transport in time instead of space—and to these two possibilities we will now turn.

2. Catalysis. There is no direct evidence as to whether muscle hemoglobin acts catalytically in the muscle or not. No specific enzymatic activity has been ascribed to it: none have been investigated. It is unfortunate that we do not even know whether it is a more effective accelerator of the oxidation of fats than blood Hb, or how the catalase activites of the two pigments compare.

In one group of properties MHb occupies a strikingly intermediate position between Hb and oxidase systems. There is an often-remarked analogy between the reversible binding of oxygen by blood pigments and of a substrate by its enzyme according to the Michaelis theory. The geometrical configurations are similar, and a number of physico-chemical properties may be quantitatively compared. Four of these are listed in table 3 (to which the corresponding figures for catalase have also been added). It is seen that MHb is more like the oxidase than Hb in three out of four of these properties: its oxygen affinity, its speed of combination, and its relative CO/O_2 affinity. Finally, MHb is much more easily oxidized to the met form than Hb; since hemin catalysts probably owe their activity to their easy alternation between the bivalent and trivalent states, the muscle pigment in this property too is more like the enzyme than is the blood pigment. It appears to

be a sort of half-way station or connecting link between oxygen carriers and oxygen catalysts.

It is just conceivable that MHb might suddenly reduce its oxygen affinity at a moment of oxygen need, perhaps by the alteration of surface forces associated with contraction, in this way providing an extra supply just when it is wanted. This would be catalysis of a rather special sort. It is, however, highly improbable and there is some evidence against it.

TABLE 3*

Comparison of some properties of blood hemoglobin, muscle hemoglobin, oxidase, and catalase

Neutral pH, room temperature, figures only approximate

PROPERTY	Hb HORSE	MHb HORSE	OXIDASE YEAST, COCCUS, ETC.	CATALASE, HORSE LIVER
Substrate concentration at ½ saturation, millimols	1.7×10^{-2}	1.5×10^{-3}	5×10^{-5}	2.5×10^{-1}
Combination velocity constant, 1/sec. millimol	7×10^{3}	1.7×10^{4}	$>2.3 \times 10^{6}$	$>7.6 \times 10^{3}$
Dissociation velocity constant, 1/sec	c. 40	c. 40	>c. 5	3×10^{5}
O_2/CO at equipartition (M)	500	18	1/10	
Oxidation state of Fe	Bivalent, oxidizable	Bivalent, easily oxidizable	Bivalent and trivalent alternating ?	Bivalent and trivalent alternating ?

* The data for this table were obtained from the following sources: Hb affinity (9); MHb affinity (44); oxidase affinity (45); catalase affinity (11); velocity constants: Hb, MHb (24); Atfo (45); catalase (50); M (2, 43).

3. *Store.* The store theory is supported both by the qualifications of the pigment and by direct evidence. An effective store must have *a*, sufficient capacity; *b*, be able to load and unload its oxygen at suitable oxygen tensions, and *c*, be able to do it quickly.

a. The storage capacity must be measured in terms of the oxygen requirements. As a typical example of a red muscle let us take the normal dog's heart. Its MHb concentration is about 0.5 per cent, the oxygen capacity of which is about 0.008 cc. per gram of tissue, while the oxygen consumption is about 4.5 cc. per gram per hour; the entire

stored oxygen would be used up 4.5/0.008 or 560 times an hour or once in about 7 seconds. In extreme activity, the metabolic demands may be ten times this amount, when the oxygen store would last only about half a second, which is just about sufficient to tide the muscle over from one contraction to the next. The store must therefore be a short time one.

If the output of the organ were to remain about the same but the frequency of contraction were to increase, the storage demands would diminish until ultimately the oxygen in solution might satisfy them. This would account for the absence of the pigment in the rapidly-beating hearts and in wing muscles of insects, and would account for its presence in slow-beating hearts, in the diaphragm, and in running muscles requiring maintained repetitive activity. An exceptionally high concentration occurs in the muscles of the seal, where according to Theorell (44) the muscle press juice may contain from 5 to 10 per cent of pigment. This is something like ten times the concentration normally found in mammals, and should provide an oxygen supply sufficient to last for half a minute or so of moderate activity. In this animal, then, the store function may have been extended to far longer periods than that between one contraction and the next. This fits in well with the animal's known ability to swim for some time under water.

b. The affinity of MHb for oxygen lies between that of blood Hb and the oxidases, so that it is well adapted to take up oxygen from the one and give it out to the other. This may be seen from the dissociation curves of figure 1, which has been taken from the paper by R. Hill (1936), extended from Barcroft. At a venous pressure of 40 mm. Hb is 66 per cent saturated; the MHb at this tension is 94 per cent saturated, so that even at the venous end of the capillaries the store will be nearly full, though the blood supplies are seriously depleted. But the store to be of any use must be emptied as well as filled, i.e., the cells must be able to take up oxygen at an oxygen pressure so low that even MHb has given up a considerable fraction of its oxygen. This is just what Warburg and Kubowitz have found them capable of doing (45). They found for yeast at 10° that 65 per cent of full aerobic respiration remained at a pressure of under 0.5 mm. Hg, and it is probable that even here the limiting factor was not the affinity of oxidase for oxygen but diffusion within the cell. Experiments by Keilin (19) on heart muscle preparations indicate that for this tissue also the oxidase can function at pressures below 5 mm. It can be seen from figure 1 that at this

pressure the pigment has already given up 40 per cent of its total store, while at 1 mm. pressure it has yielded up over 65 per cent.

c. The rates of reaction with oxygen are rapid enough to enable MHb to load and unload very rapidly, the times for half reaction being of the order of 0.001 and 0.01 second respectively under physiological conditions.

Direct observation of the store function in vivo. It is possible to see and record the use of muscle hemoglobin as a store in the soleus muscle

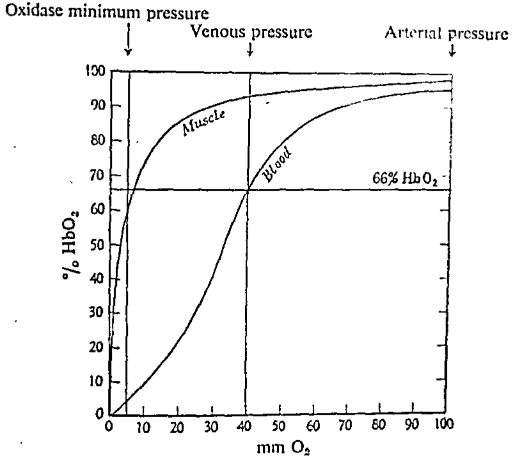


Fig. 1. The relationships of the dissociation curves of muscle hemoglobin and of blood to the minimum oxygen pressure required by the tissue. (R. H.: 15, 1936) (reproduced by permission).

of the cat, unexcised and with the nerve and blood supply intact (25). In these experiments light was passed through the muscle transversely and the colour of the emergent beam analyzed by means of a differential photocell colorimeter, by means of which the degree of oxygenation of the MHb could be continuously recorded, the galvanometer deflections of the colorimeter being photographed on moving bromide paper. When the muscle was tetanized, it instantly began to change its colour, the MHb losing from 9 to 40 per cent of its oxygen in the first second of

contraction, even though the blood supply was left intact. Thus in the normal, intact muscle the blood was unable to supply the instantaneous oxygen demands of the tissue during contraction, and the store was drawn upon. As soon as the muscle was allowed to relax, it began to regain its redness, the store being replenished. A tracing of a typical record is reproduced in figure 2.

A store can operate either to smoothe out a fluctuating supply, or it can enable the organ possessing it to make fluctuating demands under conditions of steady supply. Muscle hemoglobin appears to act primarily in the second of these two ways, at any rate in the soleus of the

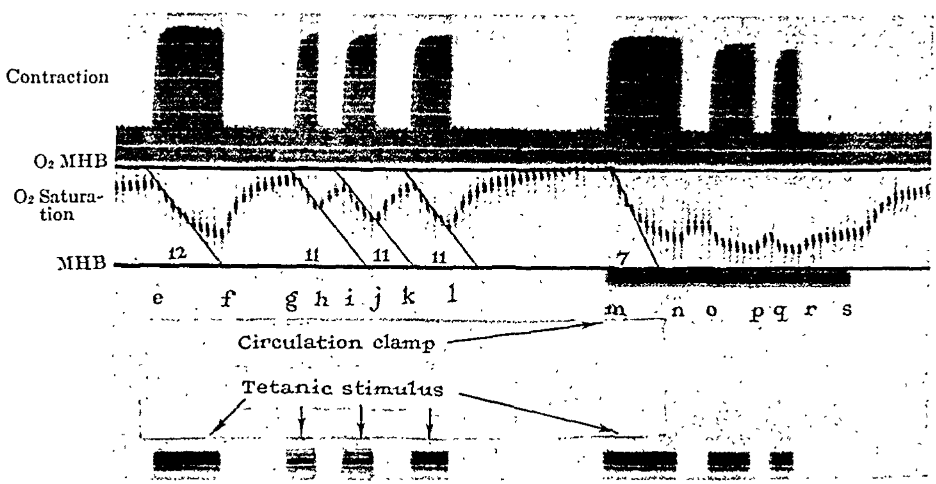


Fig. 2. Oxygen storage by muscle hemoglobin in the soleus of the cat. At the left the circulation is intact, at the right it is clamped off in the descending aorta. The interruptions in the oxygen saturation curve are 1 second time marks. (*G. M. 25 reproduced by permission.*)

cat. In this preparation, maximum contraction of the muscle showed little tendency to reduce the flow through it, as could be seen by applying an external clamp on the supplying artery, and observing the much more rapid oxygen depletion (right hand side of fig. 2). It is not the purpose of this review to enter into the much-debated question of blood flow through muscle during contraction, though the very recent work of H. Barcroft on the intact human gastrocnemius under voluntary sustained contraction provides convincing evidence that a stoppage under some circumstances does take place. In such muscles the store acts in both ways, for the supply fails just as the need is greatest, and we have both intermittent supply and intermittent

demand. The value of the store depends upon the speed with which oxygen is demanded when the muscle contracts. This will be considered in the next section.

5. RAPID CHEMICAL CHANGES IN MUSCLE. *The instantaneous measurement of oxygen consumption.* Quite apart from its physiological usefulness, muscle hemoglobin has the unique distinction of being a naturally occurring intracellular oxygen tension indicator with a very short time of response (about 1/100 sec.) Three properties fit it for this rôle: *a*, its colour change on oxygenation, enabling optical methods of measurement to be used, which involve no damage to the cell; *b*, its working range from a fraction of a millimeter of oxygen tension up to 20 or 25 mm., which is the working range through which the muscle itself operates;[6] *c*, its ability to load and unload quickly, thus bringing it into rapid equilibrium with its oxygen environment These put a new tool into our hands, for they enable us for the first time to follow the detailed time course of oxygen utilization inside a living cell, during normal and extraordinary metabolic activity. Previously available *time-sensitive* methods of studying cell metabolism (electrical, thermal, morphological, etc.) have not been *chemically-specific:* the measured quantities have been the algebraic sum of several phenomena. Manometric and gas exchange methods, on the other hand, require several seconds at the very least for equilibration, the speed being limited by diffusion processes in the cells, through the bathing medium and across phase boundaries. Sudden freezing and subsequent analysis involves irreversible destruction of the tissue. The MHb method is specific for oxygen, but it is also rapid.

Using this "metabolic movie camera," (25) we find that in the cat's soleus increased oxygen consumption gets under way in less than ⅓ of a second after the start of contraction, and has reached its maximum rate before 1 second has elapsed, these figures being the minimum response time and the minimum measurement time of the instrument respectively, so the times may be much shorter even than this. The rapid uptake can be seen from the abrupt dive of the oxygen curve at the onset of each contraction in figure 2. This "quick oxygen," furthermore, constitutes the great bulk of the total oxygen required by the contracting muscle; this is deduced from the fact that the rate of

[6] The high oxygen affinity of MHb has also been made use of by R. Hill (16) for detecting small amounts of oxygen produced by isolated chloroplasts undergoing photosynthesis.

oxygen consumption increased tenfold during contraction, but returned nearly to its resting value ten seconds after the end of tetanus.

Implications for muscle metabolism. There are two ways in which we might explain the rapid oxygen consumption. The first is by means of the classical theory, according to which the anaerobic contraction process is followed by oxidative recovery and restitution; the second is by postulating a direct oxidative mechanism in addition to the anaerobic one. It is not easy to reconcile the slow aerobic recovery heat (on the classical theory this is the time during which the oxygen is consumed) with the very rapid rate of oxygen uptake found in the MHb experiments. In frog sartorius muscle at low temperatures the recovery heat is only half liberated in ten minutes, and very recent work of D. K. Hill (13a) has shown that under these conditions the oxygen uptake runs parallel to the recovery heat production. The difficulty is not necessarily an insuperable one, however, for frog muscle at 0° and mammalian muscle at 37° are very different tissues, and an increase in reaction rates of several hundred fold is quite conceivable. Cattell and Shorr (7a) report that in dog's scalenus muscle at 37°, the recovery heat is very rapid, reaching its maximum rate in a few seconds, and could only be separated from the initial heat by the method of curve analysis. Is it possible that if we could replace isolated portions of scalenus muscle by uninjured muscle with circulation intact, the few seconds would be reduced to a fraction of a second?

More difficult to explain away is the direct chemical evidence against the classical view, which has been accumulated in the last few years by Sacks and Sacks. The recent paper by Sacks (36) summarizes their present position. They have studied the recovery rates of the three chemical processes which have been associated by biochemists with anaerobic muscular activity, after a "steady state" period of stimulation. The results are comparable with those of the MHb experiments, for they were obtained on the gastrocnemius muscle of the cat, which had been contracting under relatively normal conditions until the moment of freezing of the tissue for chemical analysis. The three reactions studied (the removal of lactic acid, the resynthesis of phosphocreatine, and the resynthesis of adenosine triphosphate) were each of them found to require several minutes, and were therefore much too slow to play a significant rôle in rapid oxidative recovery. The adenosine triphosphate resynthesis was somewhat less satisfactory than that of the other two reactions, as the changes measured were very much smaller. The acceptance of these results does not necessarily mean the

abandonment of the classical theory of anaerobic contraction and oxidative recovery. It does, however, mean something nearly as catastrophic, namely, that none of the reactions of anaerobic muscle biochemistry seem to be of much importance for aerobic activity.

The theory that a direct oxidative process provides the energy for contraction was proposed by Sacks in 1932. Since the ability of muscles to operate for a time anaerobically is unquestioned, this hypothesis requires a dual mechanism: one, a normal, aerobic, quick, efficient mechanism; the other, an emergency, oxygen debt mechanism, presumably of lower efficiency, called into action when there is an oxygen lack. In terms of the well-known analogy, the muscle can run off its batteries if it has to, charging them up afterwards, but it prefers to operate by direct drive if it can.

This is, in a way, a negative theory, and it is inherently improbable. It attacks—though not for the first time—all of the three chemical reactions proposed by muscle biochemistry as the principal energy-producing processes of aerobic contraction, but it proposes no alternative chain. For "direct" we might as well read "unknown but rapid." And it is unlikely, for striated muscle gives no hint of this dual nature in its histological structure or in its chemical composition. If two mechanisms have developed side by side, is it not strange that throughout the animal kingdom we have not a single example of a muscle in which the inefficient anaerobic process has been allowed to degenerate?

But neither of these objections should be taken too seriously. They are philosophic rather than scientific, and are merely a reflection of our lack of knowledge. They but emphasize the fact that the fundamental problem of muscular physiology, the mechanism of transfer of energy from the chemical to the mechanical system, is still quite unsolved. The relation of aerobic to anaerobic metabolism will be an inherent part of the solution to that problem.

SUMMARY

Muscle hemoglobin occurs in those muscles requiring vigorous repetitive activity, which must be maintained. Its concentration increases with age and activity. It can be obtained in crystalline form, is a true hemoglobin, but differs from blood hemoglobin quantitatively in the following ways: 1, its absorption bands are displaced to the red; 2, it has a very high oxygen affinity, and a hyperbolic dissociation curve; 3, it has only one iron atom per molecule; 4, it has a very small Bohr effect; 5, it has a low affinity for CO relative to O_2.

There is no evidence that muscle hemoglobin acts catalytically, though its properties are intermediate between those of the blood hemoglobins and oxidation enzymes. It does act as a short-time oxygen store, tiding the muscle over from one contraction to the next.

As an indicator of oxygen tension in the cell, muscle hemoglobin enables us to follow the time course of oxygen uptake during rest, contraction, and recovery. It is found that oxygen demand rises instantaneously during contraction, the bulk of all the oxygen needed being utilized in less than one second from the time the work is performed.

REFERENCES

(1) Anson, Barcroft, Mirsky and Oinuma. Proc. Roy. Soc. B **97**: 61, 1925.
(2) Barcroft. The respiratory function of the blood. Vol. II, Hemoglobin (1928).
(3) Barcroft. The architecture of physiological function (1934) Chapter V.
(4) Camus and Pagniez. Compt. rend. Acad. Sc. 1010, 1902.
(5) Camus and Pagniez. Compt. rend. de la soc. de biol. **57**: 121, 1904.
(6) Camus and Lucet. Rec. de med. vet. 123, 1911.
(7) Carlstrom. Skand. Arch. f. Physiol. **61**: 161, 1931.
(7a) Cattell and Shorr. Am. J. Physiol. **115**: 26, 1935.
(8) Forbes and Roughton. J. Physiol. **71**: 229, 1931.
(9) Ferry and Green. J. Biol. Chem. **81**: 175, 1929.
(10) Gunther. Virchow's Arch. **230**: 146, 1921.
(11) Haldane, J. B. S. Proc. Roy. Soc. B **108**: 559, 1931.
(12) Haurowitz. Ztschr. Physiol. Chem. **232**: 125, 1935.
(13) Hektoen, Robscheit-Robbins and Whipple. J. Infect. Dis. **42**: 31, 1928.
(13a) Hill, D. K. (To appear shortly.)
(14) Hill, R. Nature **132**: 897, 1933.
(15) Hill, R. Proc. Roy. Soc. B **120**: 472, 1936.
(16) Hill, R. Nature **139**: 880, 1937.
(17) Jonxis. Personal communication.
(18) Keilin. Proc. Roy. Soc. B **98**: 312, 1925.
(19) Keilin. Proc. Roy. Soc. B **106**: 442, 1930.
(20) Keilin and Hartree. Proc. Roy. Soc. B **122**: 298, 1937.
(21) Kennedy and Whipple. Am. J. Physiol. **76**: 685, 1926.
(22) Kühne. Virchow's Arch. **33**: 79, 1865.
(23) Lankester. Pflüger's Arch. **4**: 315, 1871.
(24) Millikan. Proc. Roy. Soc. B **120**: 366, 1936.
(25) Millikan. Proc. Roy. Soc. B **123**: 218, 1937.
(26) Morgan. J. Biol. Chem. **112**: 557, 1936.
(27) Mörner. Nord. Med. Arkiv., no. 2, 1897.
(28) Needham. Physiol. Rev. **6**: 1, 1926.
(29) Ray and Paff. Am. J. Physiol. **94**: 521, 1930.
(30) Redfield. Biol. Rev. **9**: 175, 1934.
(31) Roche and Bendrihem. C. R. Soc. biol. **107**: 639, 1931.
(32) Roche. C. R. Soc. biol. **110**: 1084, 1932.

(33) ROCHE. Bull. Soc. Chim. biol. 15: 110, 1933.
(34) ROCHE. Essai sur la biochemie générale et compare des pigments respiratoires, 1935.
(35) ROUGHTON. Proc. Roy. Soc. B 115: 451, 1934.
(36) SACKS. Am. J. Physiol., 122: 215, 1938.
(37) SCHÖNHEIMER. Ztschr. physiol. Chem. 180: 144, 1929.
(38) SHENK, HALL AND KING. J. Biol. Chem. 105: 741, 1934.
(39) SVEDBERG. Koll. Ztschr. 85: 119, 1938.
(40) THEORELL, A. H. Bioch. Ztschr. 252: 1, 1932.
(41) THEORELL, A. H. Bioch. Ztschr. 268: 46, 1934.
(42) THEORELL, A. H. Bioch. Ztschr. 268: 55, 1934.
(43) THEORELL, A. H. Bioch. Ztschr. 268: 64, 1934.
(44) THEORELL, A. H. Bioch. Ztschr. 268: 73, 1934.
(45) WARBURG AND KUBOWITZ. Biochem. Ztschr. 214: 5, 1929.
(46) WATSON. Biochem. J. 29: 2114, 1935.
(47) WHIPPLE. Am. J. Physiol. 76: 693, 1926.
(48) WHIPPLE. Am. J. Physiol. 76: 708, 1926.
(49) WHIPPLE AND ROBSCHEIT-ROBBINS. Am. J. Physiol. 78: 675, 1926.
(50) ZEILE AND HELLSTROM. Ztschr. physiol. Chem. 192: 171, 1930.

Earlier reviews, and reference papers

Early literature, up to 1921, GÜNTHER (10).
Occurrence relative to cytochrome, KEILIN (18).
Red and white muscle, D. M. NEEDHAM (28).
All important properties, THEORELL (41–44).
Physiological significance, Chapter V, BARCROFT (3).
Storage function, muscle metabolism, MILLIKAN (25).

THE ARCHITECTURE OF VIRUSES

W. M. STANLEY

Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey

The atomic theory of matter, the germ theory of disease, and the cell theory of life may appear at first to be quite diverse and unrelated. However, it will be the purpose of this review, not only to present the recent advances in virus research, but also to indicate that the successful continuation of this work may be dependent upon an intimate knowledge of the three theories just mentioned and even, perhaps, upon their integration into a new and unified philosophy. The atomic theory, probably first stated before 1000 B.C., has been subjected to continual modification in detail as a more exact knowledge of atomic structure has been gained, yet the original and basic concept of matter as a discontinuous rather than as a continuous or homogeneous phase has remained unaltered. The germ and cell theories have been subjected to less modification than has the atomic theory, perhaps because of their more recent origin, hence with time and new knowledge it would not be unexpected if they too should require further alteration. It is an unfortunate fact that knowledge gained before its due time frequently results in a strong desire to overthrow useful theories, and it is only later, following understanding, that reconciliation and accord are achieved. For example, it is known that many present day facts are entirely inconsistent with the atomic theory as stated by Dalton in 1808, yet the evolution of the theory has been such as to account for these facts and to provide today a valid and coherent atomic theory. The newer knowledge of viruses may appear inconsistent with present day theories, yet there is every reason to believe that a more complete understanding of the structure or architecture of viruses will permit the blending of hypotheses and facts into a unified philosophy without the overthrow of time-honored theories.

The word "virus" was originally used only in the singular and meant a poison such as a snake venom. Later it was used to denote infectious disease-producing entities without regard to their nature, and more recently it has been applied only to those infectious agents capable of

passing through filters that retain ordinary bacteria. Today, properties ascribed to viruses include not only their ability to pass fine membranes but also a set of general properties which emphasize the intimate relationship that exists between viruses and their host cells. These include the fact that viruses reproduce, but do so only within certain living cells, the fact that during reproduction they may change or mutate, the fact that many virus-infected cells contain inclusion bodies, and the fact that most, but not all, virus diseases are followed by a lasting immunity in recovered hosts. Tobacco mosaic, which was the first of the agents now known as viruses to be discovered, was shown to be filterable by Iwanowski (1) only 47 years ago and was recognized as a new kind of infectious agent by Beijerinck (2) 41 years ago. Other viruses to be recognized since then include those responsible for the foot-and-mouth disease of cattle, louping ill of sheep, hog cholera, rabies, dog distemper, fowl pox, smallpox, psittacosis, yellow fever, St. Louis encephalitis, horse encephalitis, poliomyelitis, fever blisters, certain types of tumorous growths in fowls and other animals, various yellows and mosaic diseases of plants, and even the unusual colors produced in the flowers of plants which in tulips is called tulip break. Still more viruses are being discovered from time to time.

It should be emphasized that viruses were first recognized and have continued to be recognized only by means of their biological activity, *that is to say, by the diseases that they cause.* They were regarded merely as infectious disease-producing principles and, with the exception of the elementary bodies of vaccine and other viruses which are as large as accepted organisms, attempts to isolate a virus in tangible form had resulted in failure, hence little or nothing was known of their true nature. An element of mystery tended to surround them, and they were regarded variously as invisible forms of ordinary bacteria, as protozoa, as some new type of invisible living organism, as enzymes, as unusual products of cellular metabolism, as toxins, and as different kinds of chemical principles. However, in 1935 it was found possible to isolate from mosaic-diseased plants by chemical means a material which appeared to be a high molecular weight protein and which was distinguished by the fact that it possessed the properties of tobacco mosaic virus (3). This was followed by the isolation, by the same or similar chemical methods or by means of differential centrifugation, of similar high molecular weight nucleoproteins possessing the properties respectively of aucuba mosaic (4), enation mosaic (5), tobacco ring spot (6), latent mosaic of potato (6–8), severe etch (6), Shope rabbit papil-

loma (9), bushy stunt of tomato (10), cucumber mosaic 3 and 4 (11), and tobacco necrosis (11a, 11b) viruses, and of a staphylococcus bacteriophage (12). The presence of high molecular weight protein material in very active preparations of chicken tumor I (13), equine encephalitis (14), and foot-and-mouth disease viruses (15) has also been demonstrated, although Claude's (16) isolation of a fraction from normal chick embryo that is quite similar to the purified chicken tumor I preparation casts some doubt upon the significance of the results in the cases of the first two viruses. The isolation of the different high molecular weight nucleoproteins was of importance because at last tangible materials possessing quite definite physical and chemical properties were available for study, and thus the possibility was offered of correlating virus activity with such properties. Although some of the mystery surrounding viruses was removed by the isolation of the nucleoproteins carrying virus activity, the isolation really represented but a small step towards the solution of the problem of the ultimate nature of viruses. Protein structure may be expressed in many different ways as in hormones, enzymes, toxins, respiratory materials, and perhaps as in genes and as in protoplasm, and, since practically nothing is known about protein structure, the addition of viruses to this diverse group aided but little in the establishment of their true nature. All viruses appear to have a high molecular or particle weight, yet this fact alone can not be used as a criterion of virus activity (17, 18), for some of the virus nucleoproteins may be inactivated by appropriate treatment without changing their size greatly. However, no entity having a size smaller than that corresponding to a molecular weight of about 400,000 has been found to possess virus activity, hence it is probable that a certain amount of structure may be necessary to support such biological activity.

At the present time the solution of the virus problem appears to reside in the elucidation of the structure peculiar to materials carrying virus activity. This problem is more complex than that involved in protein structure as such, for, of the virus materials so far isolated, none have been found to yield only amino acids on hydrolysis. The simplest appear to be composed of protein plus nucleic acid, the more complex of protein, nucleic acid, and carbohydrate, and the most complex of materials indistinguishable from those found in bacteria. These results may be interpreted as indicating that viruses are protein or protein-like in nature, but there is little justification for the tendency to accept the results as demonstrating unequivocally that viruses are non-living

and are merely ordinary protein molecules similar to egg albumin. Although the physical and chemical properties of the virus nucleoproteins are similar to those of ordinary proteins, the biological properties are quite different, and it is because of the virus activity, which implies the ability to multiply and to change or mutate, that it is difficult to conclude that they are ordinary protein molecules. Despite this difficulty, the virus nucleoproteins which have been found to have the chemical and physical properties of molecules will be referred to as molecules in this review. Others may wish to refer to these same particles as organisms or cells, yet, as will be indicated in the last section, the question of nomenclature is of secondary importance. The virus activity is undoubtedly a consequence of the unique architecture of the materials that have been isolated, yet it is necessary, before proceeding with such an assumption, to ascertain whether or not it can be demonstrated that the virus preparations are essentially pure and that the virus activity is a specific property of the major component. This demonstration is dependent upon the correlation of chemical and physical properties with activity, hence virus activity and its measurement become all-important considerations and will, therefore, be discussed first.

Virus activity—its measurement. The most characteristic and at the same time the most important property of viruses is their biological activity, their infectiousness or ability to multiply or reproduce when introduced into certain living cells. It is this property that resulted in the original discovery of viruses, and it is this property, more than any other, that has caused viruses to be regarded as elementary living organisms, for the ability to multiply within an essentially non-specific and variable environment has been generally considered to be one of the essential characteristics of living organisms. This property also distinguishes viruses from ordinary protein molecules, such as those of egg albumin and of hemoglobin, or even from the biologically active enzyme proteins, such as pepsin or trypsin which have the ability of multiplication in that they can cause the autocatalytic conversion of specific inactive precursors to pepsin and trypsin. It may be noted here that, should specific virus precursors be found, the virus reaction would then resemble that of the enzymes, pepsin and trypsin.

During most of the early work with viruses, no attempt was made to measure virus activity quantitatively. The activity determinations were merely qualitative and were made to determine whether or not a given preparation could cause infection. In these tests, a number of

animals or plants were inoculated and the appearance of disease symptoms was used as an indication of the presence and transfer of virus. In most of these tests, one animal or plant was required for each successful transfer of virus and it was not known whether one or more than one infective doses of virus had been administered. Later, in an effort to secure a quantitative measure of the amount of virus present in the preparation, a number of animals or plants were inoculated with various dilutions of the preparation, some of which were sufficiently dilute so that no infection followed the inoculation. This procedure established minimal infective doses and permitted the estimation of the number of such doses in the original virus preparation. However, this method of estimating virus quantitatively was subject to great error due to the tremendous variation in the response of the test animal or plant. Although by careful standardization the method can be made reasonably accurate, in most cases no attempt was made to estimate differences in virus concentration less than that corresponding to a 10-fold difference, viz., one power of ten. It is obvious that in the isolation and fractionation of virus preparations there could be differences of several hundred per cent in the virus content of different samples that would go unrecognized. The fact that it was difficult, and in many instances impossible, to follow the major portion of the virus in preparative or fractionation work was, perhaps, one of the severest handicaps that was imposed upon the earlier workers.

Calmette and Guérin (19) in 1901 noted that the number of lesions produced on a rabbit following inoculation of vaccine virus could be used as a measure of the potency of the virus preparation, but it was not until the years immediately following 1929, when Holmes (20) reported similar results with tobacco mosaic virus on certain plants, that the great usefulness and accuracy of the local lesion method of virus estimation was demonstrated. Since each primary lesion is indicative of the successful transfer of virus, and since it is possible to secure many lesions on a single animal and literally hundreds of lesions on a single leaf, it is obvious that one individual giving such a response can yield information obtainable only with hundreds of individuals giving a systemic response. The utilization of this fact has been of signal importance in plant, as well as in animal, virus research. The local lesion method of Holmes has been modified by Samuel and Bald (21) and by Youden and Beale (22) and standardized by Loring (23), so that today there is a method available for the estimation of the concentration of tobacco mosaic virus that is sufficiently accurate so that differences in

virus concentration of 10 per cent or greater may be detected without undue effort. Similar methods are used by Rous and co-workers (24) in the titration of the Shope rabbit papilloma virus, although the accuracy is probably not as great as that achieved with tobacco mosaic virus. It should be emphasized that the methods under discussion are for the estimation of the relative amounts of virus present and not for the estimation of absolute amounts of virus. Although Parker and Rivers (25) have used a dilution end-point method in estimating vaccine virus with success, in most cases attempts to estimate the absolute amount of virus present in a sample by the titration end-point have yielded very erratic results. In the case of tobacco mosaic virus, a single preparation may yield dilution end-points differing by a factor of a thousand or more, depending upon the conditions and the test plant that are used. Even when tests are carried out at the same time and with the same batch of test plants, 10-fold differences in the dilution end-point are encountered.

It was hoped that with the isolation of tangible materials carrying virus activity it would be possible to inoculate solutions containing given amounts of such materials and secure definite numbers of lesions. Although the order of magnitude of the number of lesions given by a definite amount of virus is known, viz., a solution containing 10^{-5} grams of carefully prepared tobacco mosaic virus nucleoprotein per cubic centimeter in 0.1 M phosphate buffer at pH 7 will usually yield from 50 to 150 lesions per leaf on *Nicotiana glutinosa* L., the method can not be used as an absolute measure because of the variation in the number of lesions obtained. Under adverse conditions, such a sample may give in the neighborhood of 50 lesions per leaf, whereas under especially good conditions, 150 or more lesions may be obtained. Since a similar variation in the number of lesions may result from differences of 100 per cent or more in the actual concentration of virus, it is impossible to be certain from the absolute number of lesions obtained whether any variation is due to a difference in the amount of virus or to the conditions under which the test is carried out. There is today, therefore, no way of estimating directly and accurately absolute amounts of tobacco mosaic virus by activity measurements. It is necessary to estimate the virus by comparing the activity against that of an arbitrarily selected standard under well defined conditions. Since it is known that differences in virus activity of 10 per cent or more may be readily detected, the sole difficulty in the measurement is the selection of the standard used for comparison. Many different viruses are known to lose activity rapidly

when allowed to stand, and recent evidence indicates that even the supposedly very stable tobacco mosaic virus is actually unstable in that a loss in activity can be demonstrated if the virus is allowed to stand over a sufficiently long time. It is obvious, therefore, that at best a preparation gradually changing in activity must be used as a standard. This is not a serious difficulty in the case of viruses as stable as that of tobacco mosaic, for a period of several weeks is required before the change can be detected. A more serious and practical difficulty is the fact that different methods of preparation yield samples that differ widely in activity. Since some methods of preparation obviously cause the inactivation of much virus and yield preparations of low specific activity, the most active virus preparation obtainable should be used as the ultimate standard for comparison. In the case of tobacco mosaic virus, it has been found that a few careful ultracentrifugations of the untreated juice from frozen badly diseased Turkish tobacco plants yield preparations which do not differ measurably from each other in activity and which on a protein basis possess activity that is not measurably different from that of the virus in the untreated juice (26). Such preparations possessing this maximum activity are referred to as "fully active" virus and should be used as the standard for comparison.

In the early work with tobacco mosaic virus, some preparations were obtained by subjecting the virus to strong concentrations of ammonium sulfate at room temperature and, because the methods of measuring virus activity then in use were not sensitive, it was not found that such preparations varied widely in activity depending upon the extent and nature of the chemical treatment. Recent work has demonstrated, however, that such treatment may yield preparations possessing only 1 per cent of the original activity. Such almost completely inactivated preparations may still be crystallized, will yield liquid crystalline solutions, and in many other respects are quite similar to fully active preparations. Whether such preparations consist of a mixture of a small amount of fully active virus plus a large amount of completely inactive virus, or of an essentially homogeneous phase of units, each of which possesses a greatly lowered activity, is not known, but it seems likely that the former is the case. The failure to recognize the difference between partially active and fully active preparations has resulted in many difficulties and misunderstandings, and it is to be hoped that the experience with tobacco mosaic virus will aid in the research with other viruses, where in most cases the situation will be even more difficult. For example, Loring (27) has found that merely allowing latent

mosaic virus to stand in the presence of 10 per cent ammonium sulfate for one day at room temperature causes a 50 per cent reduction in the number of lesions given by the sample, and that isolation of the virus from juice by means of 5 precipitations with salt yields a preparation having only about 10 per cent of the activity of a preparation obtained from the same juice by ultracentrifugation. Similarly, the writer has found that one precipitation of tobacco ring spot virus with 30 per cent ammonium sulfate results in a loss of about 50 per cent of the activity, and that solutions of this virus in water lose activity rather rapidly, even when held at 2°C., whereas solutions in 0.01 M phosphate buffer at pH 7 retain their activity fairly well, provided they are held at 2°C. However, even with these rather unstable viruses it has been found possible to obtain preparations having a high and uniform activity and probably consisting, therefore, of fully active virus. It may be concluded that, while it is not possible to estimate the absolute amount of virus present in a sample solely by means of its activity, methods are available for detecting small differences in the concentration of those viruses giving a local lesion response. In such cases it is possible not only to differentiate between preparations of low and high activity, but also to establish, as the standard for comparison, a preparation which may be referred to as fully active virus.

Correlation of virus activity with chemical and physical properties. The demonstration, beyond a reasonable doubt, that the virus preparations which have been isolated, or at least the major component in each of the preparations, actually consists of virus is of paramount importance, for the further consideration and the acceptance of the preparations as virus are directly dependent upon such demonstration. Although much of the work done since the isolation of the purified virus preparations has been devoted to a study of this problem, there will be presented here briefly only the more significant results, most of which have been obtained with tobacco mosaic virus. This virus has one of the widest host ranges known, for 46 different species of plants representing 14 widely separated families are susceptible to the mosaic disease (28). Although attempts to obtain purified preparations from all of these have not been made as yet, virus preparations possessing essentially the same chemical, physical, and biological properties have been obtained from different batches of diseased Turkish tobacco, Burley tobacco, tomato, phlox, spinach, petunia, and nightshade plants. However, the yield of virus obtained was found to vary widely depending upon the host. Turkish tobacco plants gave a yield

of 2 to 5 mgm. of purified virus per cubic centimeter of juice, tomato plants about 1 to 3 mgm. per cubic centimeter, and spinach and phlox plants considerably less than 1 mgm. per cubic centimeter. It should be noted that some of these plants, such as phlox and spinach, are far removed from the tobacco family. For example, no serological relationship was found by Chester (29) between the protein from normal tobacco plants and that from normal phlox plants. The fact that on infection these two different plants foster the production of the same virus is an indication that virus is not produced by the simple polymerization of serologically active normal proteins.

There is good evidence that during production in a host viruses occasionally change or mutate and give rise to new strains which may be isolated, grown, and studied apart from the parent virus (30, 31, 32). Many strains of tobacco mosaic virus are recognized and to date 4 of these have been isolated in purified form. Preparations of strains of the same virus have been found to have somewhat similar general properties, yet it was found possible to distinguish each of the 4 preparations by means of definite and characteristic chemical, physical, and serological properties (4, 5). Furthermore, the concentration reached in a given host was found to vary widely with the strain of virus (33). The amounts of the different purified viruses obtainable from a given quantity of starting material have also been found to vary widely. For example, some batches of badly diseased Turkish tobacco plants have been found to contain one part of virus per 200 parts of fresh green plant material, whereas Turkish tobacco plants diseased with cucumber mosaic 1 virus contain only about one part per million as virus. Beard and Wyckoff (9) obtained about one part per 5000 of the starting material, based on the whole rabbit, in the form of the papilloma virus, and Northrop (12) estimated that the crude culture of the staphylococcus with which he worked contained about one part per million as bacteriophage. It should be recognized, therefore, that the amount of virus occurring in a host may vary tremendously depending upon the virus and the host, some viruses occurring in amounts as great as a part per 200 and others in amounts as small as a part per million, with the possibility that other viruses may occur in still smaller amounts. In those cases where it has been found impossible to isolate weighable amounts of virus, or to demonstrate the presence of virus by immunological reactions, it is possible that the virus was extremely unstable or existed in such great dilution that it escaped detection by means other than activity measurements. The importance of considering

antigenic mass in virus immunization studies and in immunological reactions *in vitro* has been emphasized by Merrill (34). The purified preparations of the various viruses, such as latent mosaic, tobacco ring spot, bushy stunt of tomato, and the Shope rabbit papilloma viruses, and a staphylococcus bacteriophage, have been found to have quite different and highly characteristic chemical, physical, and serological properties. It may be concluded that the different viruses as well as the various strains of a given virus reach widely different concentrations in their hosts and that the respective purified preparations possess definite and highly characteristic chemical, physical, and serological properties which may be correlated with the biological or virus activity.

The purified viruses that have been isolated have been found to consist largely of protein which is susceptible to digestion with certain proteolytic enzymes and which may be denatured by appropriate treatment. In every instance so far studied, the digestion or denaturation of the protein has been accompanied by loss of virus activity and, in general, the rate of digestion or of denaturation and the rate of loss of activity have paralleled each other. The studies have included denaturation by acid, alkali, heat, dodecyl sulfate, urea, etc. It should be noted here that the purified preparations of the different viruses were found to be stable only over certain ranges of hydrogen ion concentration that were definite and characteristic for each virus. In most instances, at the same pH that caused loss of activity, there occurred a break-up of high molecular weight material into low molecular weight material that could be demonstrated by means of the analytical ultracentrifuge. It has also been found possible to inactivate some virus preparations, such as those of tobacco mosaic, cucumber mosaics 3 and 4, and latent mosaic, with nitrous acid, hydrogen peroxide, formaldehyde, or ultraviolet light without the accompanying gross change in properties that is usually referred to as denaturation. The general chemical, physical, and serological properties of the inactivated preparations are very similar to those of active preparations. For example, in the case of tobacco mosaic the preparation still consists of a high molecular weight nucleoprotein which may be crystallized and which reacts specifically with antiserum to active virus. Should it prove possible to inactivate a virus with but a very slight change in its makeup, it is possible that very sensitive chemical or physical tests might be necessary in order to detect the change. However, despite the great similarity in general properties, in every case yet studied it has been possible to demonstrate that the inactivation was accompanied by a

measurable change in one or more of the chemical, physical, or serological properties of the preparation. In the treatment of tobacco mosaic virus with formaldehyde, the inactivation was accompanied by a decrease in amino nitrogen as measured colorimetrically or by means of the Van Slyke gasometric method and by a decrease in the color developed by Folin's phenol reagent (35). Of considerable importance in connection with the correlation of chemical with biological properties are the facts that it was found possible to reactivate the formolized virus and to demonstrate that the reactivation was accompanied by an increase in amino nitrogen, as measured by the color developed with ninhydrin, and also by an increase in the color developed with the phenol reagent. This correlation indicates that the virus activity is a property of the nucleoprotein and provides information concerning the structure necessary for activity.

The action of 36 per cent urea in 0.1 M phosphate buffer at pH 7 on tobacco mosaic virus has been studied by Frampton and Saum (36) and at different hydrogen ion and salt concentrations by the writer with Lauffer (37), and the action on the same virus of 0.5 per cent or greater concentrations of sodium dodecyl sulfate at pH 8 was studied by Sreenivasaya and Pirie (38). In the two latter studies, the virus was found to be rapidly disintegrated into low molecular weight protein components free of nucleic acid with loss of virus activity, serological specificity, and ability to show stream double refraction. Frampton and Saum reported that, although low molecular weight material was found, there was no change in virus activity, a result that appears very questionable and one which it has not been found possible to confirm in the writer's laboratory. In the case of treatment with urea, the denaturation was accompanied by the appearance of sulfhydryl groups as indicated by a positive nitroprusside reaction, and the low molecular weight protein which was formed was soluble in water but insoluble in 0.1 M or more concentrated salt solution.

The fact that the virus preparations that have been isolated have consisted essentially of high molecular weight protein material has been utilized in still another type of experimental approach. The materials can be sedimented from solution by means of a high speed centrifuge because of their unusually high molecular weight, and, since they are protein and have an isoelectric point, the sedimentation of negatively charged protein, of positively charged protein, and of neutral or uncharged protein can be studied. In the case of tobacco mosaic virus, it was found that, regardless of the charge, the protein and the

agent carrying the virus activity sedimented at exactly the same rate. This is good evidence that the activity is a property of the protein, for, if the activity were due to a small amount of some material of a different size mixed with the protein, it is obvious that under one of the conditions cited the mixture would have been separated and the activity would not have been found with the high molecular weight protein. It was found possible to separate, by the high speed centrifugation of mixtures of tobacco mosaic virus with egg albumin, globin, trypsin, or pepsin, the characteristic high molecular weight nucleoprotein with unchanged activity (39). Gratia and Manil (40) obtained similar results with mixtures of tobacco mosaic virus and a bacteriophage.

Neurath and Saum (41), using the refractometric method of Lamm, found the diffusion constant of chemically purified tobacco mosaic virus to be about 3×10^{-8} cm.^{2}/second, a value corresponding to a molecular weight of about 50 million. The diffusion constant obtained by Hills and Vinson (42) by means of the Northrop-Anson diffusion cell is probably far too large, since these workers did not use sufficient electrolyte to eliminate the accelerating effect on the virus of small ions. In most of their experiments, virus was permitted to diffuse from a dilute electrolyte solution into distilled water, and in the remaining experiments, from a trypsin solution into a trypsin-free solution. Purified preparations of tobacco mosaic virus have been subjected to a careful immunological study, and when sufficiently purified it was not found possible to detect material other than the virus protein even by the sensitive precipitin and anaphylactic tests (43). Evidence of a different nature but also indicative of unusual homogeneity was provided by the results obtained by means of the analytical ultracentrifuge and the electrophoresis apparatus, for in each case a single sharp boundary characteristic of a single molecular species was obtained (44, 45). It was found impossible to separate virus activity from protein by filtration through collodion or other types of filters. The ultraviolet light absorption spectrum of purified tobacco mosaic virus preparations was found to agree essentially with the destruction spectrum of virus activity, thus indicating a close relationship between the two (46, 47).

Northrop (12) has amassed an impressive amount of experimental evidence which indicates that phage activity is a property of the high molecular weight protein isolated from lysed staphylococcus cultures. He found the protein diffused across a sintered glass membrane and sedimented in the ultracentrifuge at exactly the same rate as did the active agent. Of interest is the fact that evidence was secured which

indicates that the phage particles are considerably larger in concentrated solution than in dilute solution. The partial denaturation of phage protein preparations, by acid, alkali, heat, or enzymes, was accompanied by a corresponding loss of phage activity. The absorption spectrum of the phage protein was found to agree essentially with the destruction spectrum of phage activity. Of special significance is the fact that Northrop found the solubility of two especially active preparations of phage protein to vary only slightly with a change in the amount of solid phage present, a result indicative of unusual homogeneity.

A great amount of experimental work on the purified virus preparations has already been completed, and an even larger volume is now in progress. Still more viruses are being obtained in purified form from time to time and ever-increasing amounts of the ones already purified are being made available for experimentation. In all of the work that has been reported to the present time, or that is known to the writer, not a single bit of experimental evidence has been obtained that is incompatible with the idea that the various purified and unaltered virus preparations, or at least the chief component, actually consists of the active agent. On the contrary, there is an imposing array of evidence which indicates that several of the viruses have been isolated in an essentially pure form and that the biological activity is a specific property of the respective nucleoproteins. There is always the possibility that the virus materials may represent a unique situation and that there is something which has not been comprehended as yet. However, it seems to the writer that the time has arrived, or at least is rapidly approaching, when it can be said that virus activity is a specific property of the nucleoproteins, with the same degree of assurance as when it is said that the properties of water are those of the water molecule or that the hormone properties of insulin belong to the protein molecule known as insulin. On the basis of information now available, it must be concluded, therefore, that the different nucleoproteins represent the respective viruses in essentially pure form. The composition and structure of the purified materials become of importance, for if virus activity is a specific property it must result from a characteristic architecture. The information now available on the composition of the purified viruses will be considered in the next section.

Composition. Quantitative chemical analyses have been made on purified preparations of tobacco mosaic virus and its strains aucuba and enation mosaics, the related viruses cucumber mosaics 3 and 4, tobacco ring spot, latent mosaic of potato, bushy stunt of tomato, chicken tumor

I, and vaccine viruses and on a purified preparation of a staphylococcus bacteriophage. The values for carbon, hydrogen, and nitrogen for the plant virus preparations were about 50 per cent, about 7.5 per cent, and about 16 per cent, respectively. The phage preparation was found to contain about 41 per cent carbon, 5.3 per cent hydrogen, and 14.4 per cent nitrogen. The vaccine virus preparation contained 13.4 per cent nitrogen and 6.5 to 10.1 per cent lipoid and the chicken tumor I virus preparation only 8.6 to 9.5 per cent nitrogen and 24 per cent lipoid. Perhaps the most interesting of the analytical results are those reported for sulfur and phosphorus, for these have been found to vary considerably from virus to virus. Preparations of tobacco mosaic virus contained 0.24 per cent sulfur and 0.60 per cent phosphorus, and no significant difference has yet been noted in the cases of the strains or related viruses. Preparations of latent mosaic virus contained 1.1 per cent sulfur and 0.51 to 0.58 per cent phosphorus, those of tobacco ring spot 0.39 and 4.1 per cent, respectively, of sulfur and phosphorus, and those of bushy stunt virus 0.4 to 0.8 and 1.3 to 1.5 per cent, respectively, of the two elements. The chicken tumor I virus preparation contained only 0.7 per cent phosphorus, whereas the phage preparation contained 4.6 to 5.0 per cent phosphorus. All of the virus preparations were found to give a test for carbohydrate. The amount of the carbohydrate, calculated as glucose, was found to range from 1.5 per cent in the case of the phage preparation to about 16 per cent in the case of ring spot virus. All of the carbohydrate and phosphorus of tobacco mosaic and tobacco ring spot virus preparations can be accounted for by that isolated in the form of nucleic acid, hence these two viruses appear to be composed solely of nucleic and amino acids. With latent mosaic virus, only a portion of the carbohydrate appears to be in the form of nucleic acid, hence this virus must be composed of nucleoprotein plus additional carbohydrate. In the case of the phage preparation, only a small portion of the phosphorus appears to be in the form of nucleic acid, for the carbohydrate content is only about 1.5 per cent, an amount which would account for only about one-tenth of the total phosphorus. The distribution of the remaining phosphorus is unknown. The only virus preparations that have been found to contain lipoid are those of vaccinia and of chicken tumor I. It has not been determined whether the lipoid can be removed from the vaccine preparations with retention of virus activity, but in the case of the tumor agent attempts to remove the lipoid resulted in inactivation. It is possible, therefore, that lipoid may represent an integral component of the virus. MacFarlane and

Salaman (48) have reported that purified vaccine virus shows phosphatase and catalase activities but not dehydrogenase activity, and they consider that the enzymatic activities are specific properties of the virus. No other purified virus preparation has been reported to have such enzymatic activity (48a).

It is unlikely that studies on the composition of most of the viruses will consist of more than routine quantitative elementary chemical analyses in the immediate future, for at present most viruses are readily available only in amounts measurable in milligrams. However, there is no reason why the makeup of at least one typical virus, that of tobacco mosaic, should not be studied in great detail, for it is possible to obtain this virus nucleoprotein in 100-gram lots without undue effort. Preliminary studies have been completed (49), and to date the virus material has yielded only amino acids and nucleic acid on hydrolysis. The amino acids that have been identified include arginine, aspartic acid, cysteine, glutamic acid, leucine, lysine, phenylalanine, proline, serine, tryptophane, and tyrosine. Alanine, histidine, and glycine are either absent or occur in amounts that have not been measurable as yet. Doctor Ross has found the glutamic acid isolated from tobacco mosaic virus to have the customary rotation of naturally occurring d-glutamic acid (unpublished work). This is of interest because of Kögel and Erxleben's (49a) striking demonstration of the occurrence in cancerous tissues of glutamic and other amino acids having markedly lower optical rotations, indicative of the presence of the unnatural isomers. In view of the apparent dependence of insulin activity upon the presence of the disulfide linkage in the molecule, the distribution of sulfur in the tobacco mosaic virus preparation was studied (50). Of the 0.24 per cent sulfur usually found, about 0.18 per cent occurs as cysteine sulfur and 0.04 per cent or less as sulfate sulfur. Although methionine determinations frequently yield results as high as 0.04 per cent methionine sulfur the results are always somewhat lower following dialysis and it is doubtful if the virus actually contains methionine. Since virus activity is unaffected by reducing agents, disulfide groups are either absent or are not reduced or their reduction does not affect activity. It seems likely that all or most of the sulfur in tobacco mosaic virus occurs in the form of sulfhydryl groups, for the titration of the porphyrindin dye as described by Greenstein (50a) corresponds to 0.76 per cent cysteine (37).

Loring (51), in preliminary studies on the nature of tobacco mosaic virus nucleic acid, demonstrated not only that the acid is a true nucleic acid by the isolation of guanine, adenine, cytosine, and uridylic acid,

but also that the acid differs in certain respects from yeast nucleic acid. He found the diffusion constant of virus nucleic acid in 0.4 M borate buffer at pH 7.7 to be 0.10 cm.^{2}/day which, on the basis of a spherical molecule, would correspond to a molecular weight of about 37,000. The manner in which the protein and nucleic acid are combined in tobacco mosaic virus is not known. However, the fact that the two are immediately split apart on treatment with 5 per cent sodium hydroxide at 0°C., with 5 volumes of glacial acetic acid, with 36 per cent urea in 0.1 M phosphate buffer at pH 8, or on heating to 75°C., and that a fairly rapid disintegration occurs with 0.5 per cent dodecyl sulfate at pH 8 argues against the possibility that they are combined through stable chemical bonds. The fact that it has not been found possible as yet to secure evidence for the dissociation of the two in the presence of various concentrations of salt, after the manner in which sperm nucleoproteins are dissociated, indicates that the linkage is different and is probably somewhat stronger than the usual salt bonds. It is possible that the linkage is a very weak ester or amide bond or perhaps of the hydrogen bond type that is being postulated so freely at present in connection with protein structure (52). Bernal and Fankuchen (53) have deduced from x-ray data that tobacco mosaic virus contains equal sub-units about 22 x 20 x 20 A in size, and Astbury (54) on the basis of x-ray work with nucleic acids has suggested that this sub-unit, which would have a molecular weight of about 7000, must consist of one nucleotide combined with 54 amino acid residues. However, Ross has calculated from the analytical data on some of the amino acids occurring in small amounts that, if the virus is built up from similar repeat-units, the minimum molecular weight of this sub-unit must be of the order of 20 to 40 thousand. This value is in accord with Bernal's (55) recent estimate of 40,000, and with results obtained by means of the analytical ultracentrifuge on alkali dissociated virus, and by means of osmotic pressure and diffusion determinations on virus dissociated in 36 per cent urea, all of which indicated a molecular weight of the order of 50,000.

In the case of alkali degradation, evidence was obtained by means of the ultracentrifuge that the virus is first broken down into large units and these then continue to break up until eventually the small units are obtained. It has not been determined whether or not the large units still contain nucleic acid. Although it seems likely that a similar gradual breakdown occurs in the degradation of virus in concentrated urea solution, it has not been demonstrated experimentally as yet, although such studies are in progress. In no instance, other than

in Frampton and Saum's work which has not been confirmed, have the breakdown products been found to possess virus activity. Furthermore, no convincing evidence has been obtained for the reversal of the degradation process with the regaining of virus activity, although in view of the reversal of denaturation in the cases of hemoglobin and of the protein enzymes, trypsin and pepsin, it would not be unreasonable to expect that eventually conditions for the reversal in the case of viruses may be achieved. Steinhardt (56) found that the half molecules of hemoglobin obtained in 36 per cent urea still retained their characteristic absorption spectra and gas capacities and affinities. Unfortunately, it is not possible to measure virus activity in the presence of concentrated urea, but measurements made on virus degraded in urea immediately following dilution with either water or 0.1 M phosphate buffer at pH 7 showed no activity. The results indicate that the virus activity is not due to a dissociable prosthetic group, but rather to a unique architecture that is characteristic of the large molecule as a unit. Nucleic acid appears to play an important rôle in this structure, for it has been found in all of the viruses that have been purified. The nature of the combination appears to vary somewhat, for it has already been found that the nucleic acid is bound far more strongly in latent mosaic than in tobacco mosaic virus. The elucidation of the intimate structure characteristic of viruses may appear hopeless in view of the complexity and differences already found. However, if they contain somewhat similar sub-units, a study of the various degradation products should yield information concerning the general nature of the sub-units and, although the elaboration of their detailed structure may not be achieved at an early date, it is possible that the manner in which they are combined to form the virus may be learned and with this the secret of the activity.

Crystallinity and its significance. Crystalline form and the retention of constant properties following repeated crystallization have been used as criteria of purity with such great success in the chemistry of inorganic and simple organic compounds that the layman has tended to place undue confidence in them. Henderson Smith (57) has stated that there was "attached a kind of sanctity to the word 'crystal' " and that crystallinity "was a sort of a guarantee of purity." However, chemists, and especially protein chemists, who are familiar with the great tendency of proteins to form solid solutions and to carry along impurities on crystallization, have long recognized that, while crystallinity and the retention of constant properties on repeated crystallization are very useful tests in the determination of the purity of a material, they are

not infallible tests, they are less well suited for purity tests in the case of proteins, and most certainly they are not an absolute guarantee of purity. Nevertheless, following the announcement in 1935 of the "isolation of a crystalline protein possessing the properties of tobacco mosaic virus," the fact that the material could be obtained in crystalline form was more instrumental than any other in securing the acceptance of the material as pure and as the virus, although to the writer and to many others it was perhaps the least convincing of the experimental evidence. The fact that the protein could be repeatedly crystallized with retention of constant properties was a more significant bit of evidence, but even in this first paper the possibility of a solid solution of different active and inactive proteins was mentioned. The crystallinity of the protein also caused many individuals to decide that the virus was non-living because they considered it impossible for living organisms to take on a crystalline structure. However, it has been shown that crystallinity is simply a structural regularity and that actually there need be no incompatibility between the living and the crystalline states (58). Nevertheless, if the protein had not been obtained in crystalline form it is very probable that, because of the general conception of viruses as living organisms, it would have been generally considered as a minute living organism. The crystallinity focussed attention on the other physical and chemical properties of the protein and, although some workers doubted that the virus activity was a property of the protein, the material was generally accepted as being composed essentially of large protein molecules. Because all of the evidence indicated that the virus activity was a specific property of the large protein molecules, and because, at that time, the choice appeared to be only between a living and a non-living agent, the writer joined in the acceptance of the virus as a non-living protein. However, a growing realization of the futility of attempting to distinguish between the properties of non-living and of living things and an increased store of knowledge of viruses have permitted a newer and more exact definition of the general nature of viruses, which will be considered in a subsequent paragraph. Here it may be concluded that crystallinity, of itself, offers no evidence as to the living or non-living nature of a material and is no absolute guarantee of purity, and that retention of constant properties following repeated crystallization should be regarded as merely one of a great many tests used to determine purity, any one of which may be fallible and all of which should be used before a decision regarding purity is reached.

It has not been found possible to crystallize some of the unstable

virus proteins, and it is likely that more viruses will be isolated which can not be crystallized. In the cases of unstable viruses, the continual breakdown may provide sufficient impurity to prevent crystallization, and in other cases it is possible that pure preparations may be obtained which will fail to crystallize, hence failure to obtain a virus in crystalline form should not be considered as a definite indication of an impurity. In his excellent review on the nature of viruses, Levaditi (59) points out that no virus affecting animals or man has been obtained in crystalline form and considers that the crystallinity of tobacco mosaic virus and the lack of crystallinity in the case of the papilloma virus preparations represent an important difference. It may be noted that the same difference exists in the case of tobacco mosaic and tobacco ring spot viruses, very probably because of the instability of the latter virus. If a virus preparation fails to crystallize but is found to be homogeneous by all other tests, it should be regarded as a pure material. Should the papilloma virus be found to have chemical properties similar to those of tobacco mosaic virus and to be homogeneous by all available tests, the mere lack of crystallinity would not be a valid reason for differentiating between it and tobacco mosaic virus. There is good reason, therefore, to subject the purified preparations of vaccine, Shope rabbit papilloma, chicken tumor I, and foot-and-mouth disease viruses to the various tests for homogeneity, for in every case it should be possible to determine whether or not the preparations are pure and hence represent the virus. So far, there is no good reason to attempt to differentiate viruses affecting plants from those affecting man and animals.

Tobacco mosaic and its strains, aucuba, masked, and enation mosaic viruses, the closely related cucumber mosaic 3 and 4 viruses, and bushy stunt of tomato virus and tobacco necrosis virus have all been obtained in the form of crystals readily visible under the microscope. All of the first named viruses were obtained in the form of long thin pointed needles, while the bushy stunt virus was obtained in the form of rhombic dodecahedra and the tobacco necrosis virus as thin plates. Latent mosaic of potato virus has not been obtained in the form of distinct crystals, but the pellet obtained on high speed centrifugation was found to be doubly refracting. When 1 to 2 per cent solutions of latent mosaic virus or of tobacco mosaic virus or its strains are allowed to stand, they gradually separate out into two distinct layers, the lower of which is liquid crystalline. No purified viruses other than those mentioned above have been obtained in crystalline form to date. Bernal and Fankuchen (53) made x-ray diffraction studies on oriented crystals and

solutions of virus preparations and found no differences between strains of latent mosaic virus and only doubtful ones in the case of the cucumber mosaic 3 and 4 viruses. However, they found definite differences in intensity of the intermolecular pattern in the case of the strains of tobacco mosaic virus, although the intramolecular pattern appeared to be the same. In every case the intramolecular pattern was found to be independent of the concentration of the virus while the intermolecular pattern, on the other hand, varied continuously and quantitatively with the concentration. The intramolecular pattern of the bushy stunt virus was found to be of essentially the same type as that of tobacco mosaic virus, indicative of a repeat-unit of approximately 20 x 20 x 22 Å. Bernal (55) came to the interesting conclusion that the individual molecules of tobacco mosaic virus have an internal crystalline structure, that this structure is analogous to that of other crystalline proteins, and hence that each molecule may be regarded as a crystal. Although the beautiful dodecahedric crystals of bushy stunt virus are regarded as true crystals, Bernal considers the intermolecular pattern of crystalline tobacco mosaic virus to indicate the presence of long molecules arranged with a perfect hexagonal, 2-dimensional regularity at right angles to the length but with no regularity in the direction of the length. He is of the opinion that the solid needle-shaped particles visible under the microscope which have been referred to as crystals have only this type of regularity and are therefore really in a liquid crystalline state and should be referred to as liquid or para-crystals. Wyckoff and Corey (60) have also studied the x-ray diffraction pattern of crystalline tobacco mosaic virus and, although they obtained essentially the same pattern as that reported later by Bernal, they interpreted it as resulting from true crystals.

Recently, Kausche (61) reported that he had succeeded in preparing *in vitro* the hexagonal crystals of tobacco mosaic virus, which previously had been noted only within living cells (62). Although proof of identity was not offered in the preliminary paper, it seems likely that the crystals are composed of virus and that the necessary proof will be forthcoming soon. Beale (63) had previously described the transformation of the hexagonal crystals within living cells into the needle crystals upon the addition of acid. Kausche reported that he had observed under the microscope the reversal of this phenomenon, namely, that bundles of the needle crystals fused together to form the hexagonal crystals. He considers that the molecules, the short and long fibres or filaments described by Best (64), the needle crystals, and the hexagonal

crystals form an unbroken series. The preparation of the hexagonal crystals *in vitro* should make it possible to determine whether or not they are more complex in composition than the needle crystals, a possibility which was suggested by Beale. Bernal (65) has stated that the hexagonal crystals occurring within cells "possess end as well as side faces and undeniably show 3-dimensional regularity" and Bawden (66) also considers them to be true crystals. However, Bernal and Bawden consider that the needle crystals are not true crystals and possess only a 2-dimensional regularity, hence, if the hexagonal crystals are formed from the needle crystals, a rather unusual realignment of the molecules must occur. It appears preferable to leave the question of the kind of crystallinity open for the present. Whether crystalline tobacco mosaic virus possesses a 2- or a 3-dimensional type of regularity is not important from the standpoint of the virus worker, even though it may be quite important to the crystallographer for, as mentioned above, crystallinity is not a vital issue with respect to either the purity or the nature of viruses, hence regardless of the final decision the general virus problem will remain unchanged.

Size and shape. Most of our knowledge concerning the sizes of viruses has been obtained by Elford (67) using the method of ultrafiltration analysis. However, supplementary information has been obtained by means of ultraviolet light photography, and since the isolation of purified viruses in weighable amounts the excellent method of ultracentrifugal analysis of Svedberg (68) has been used with great success and will undoubtedly be used more and more in the study of viruses. It has been found that viruses may be arranged in an unbroken series with respect to size from those, such as psittacosis virus, that are about 275 mμ in diameter, down to those, such as poliomyelitis and foot-and-mouth disease viruses, that are only about 10 mμ in diameter. It should be noted that some viruses are larger than accepted living organisms, such as agalactia of sheep, bovine pleuropneumonia, and sewage organisms, which are about 175 mμ in diameter, while other viruses are smaller than accepted protein molecules, such as those of the hemocyanin proteins. Viruses, therefore, form an unbroken series from accepted organisms to accepted protein molecules, and there is an overlapping at either end. A given virus appears to have a definite and characteristic size regardless of its source, and the strains of a given virus appear to have essentially the same size. Another point which should be noted is that viruses exhibiting similar clinical behaviors, such as vesicular stomatitis and foot-and-mouth disease viruses, may

nevertheless vary widely in size. The measurements of the sizes of viruses by ultrafiltration, which constituted the earliest reasonably exact physical measurements on viruses, and the demonstration that some viruses were smaller than protein molecules and that viruses differed in mass by a factor of about 10,000 represented most important advances.

In most of the work on the estimation of the sizes of viruses, it has been tacitly assumed that the virus particles were essentially spherical in shape. Recently, however, it was demonstrated that some viruses are very asymmetrical in shape (69). The earliest indication of the asymmetry of a virus was obtained by Takahashi and Rawlins (70), who noted that double refraction of flow was exhibited by the juice from a plant diseased with tobacco mosaic virus but not by the juice from normal plants. In view of Freundlich's (71) work on the vanadium pentoxide sols and because of the nature of the double refraction of flow, they concluded that tobacco mosaic virus or some material regularly associated with it was composed of rod-shaped particles. Since that time and following the isolation of the virus in purified form, an imposing mass of evidence of a more or less indirect nature has been obtained, chiefly by Lauffer (72), that tobacco mosaic virus consists of molecules having a cross section of about 12 mμ and a length of the order of 400 mμ. The strains of tobacco mosaic virus, the related cucumber mosaic 3 and 4 viruses, severe etch virus, and latent mosaic of potato virus have also been found to have similar rod-like shapes. Direct and striking confirmation of this general conclusion was recently achieved when, by means of the electron microscope, Kausche, Pfankuch, and Ruska (72a) found the probable cross section and length of tobacco mosaic virus to be about 15 mμ and 300 mμ, respectively. Tobacco ring spot, rabbit papilloma, vaccinia, and bushy stunt of tomato viruses have not been found to show double refraction of flow and hence probably have relatively symmetrical shapes. The marked asymmetry of some of the viruses has thrown doubt upon sizes estimated by ultrafiltration or by ultracentrifugation, since few studies have been made on the manner in which rod-shaped particles filter or sediment. However, in the case of tobacco mosaic virus, Lauffer (73) has shown that different methods of estimating molecular weight from viscosity, sedimentation and diffusion data, one of which is independent of the shape of the molecules, give values that agree closely. The cross section of the molecule, calculated from the length to width ratio of 35:1 obtained from viscosity studies and the molecular weight of about 50×10^6,

is 12.3 mμ, which is in agreement with the results of ultrafiltration, if it be assumed that the width of the particle represents the limiting dimension. This value for the cross section is approximately the same as that of 15.2 mμ suggested by Bernal on the basis of x-ray data and that of 12.5 mμ suggested by Langmuir and Schaefer (74) as a result of studies on monolayers of the virus. Smith and MacClement (75) have reported that tobacco mosaic virus can be drawn through a membrane having an average pore size of only 13 mμ by means of an electrical field. Nothing is known about the relationship of particle size to pore size under such conditions, but, if it be assumed that such pores represent the minimum cross section of the virus, a size in agreement with that mentioned above is obtained. It has been demonstrated, therefore, that viruses vary in shape and in size from psittacosis and vaccine viruses with essentially spherical shapes and diameters of about 275 and 175 mμ, respectively, through a gradual series of viruses of fairly well established sizes but of unknown shapes; to tobacco mosaic virus with a molecular weight of about 50×10^6, a width of about 12 mμ, and a length of about 400 mμ; on through a group of small spherical viruses such as those of the rabbit papilloma, tomato bushy stunt, and tobacco ring spot, having diameters of 40, 28 and 19 mμ, respectively; down to the very small poliomyelitis and foot-and-mouth disease viruses having molecular weights of 700,000 and 400,000, respectively, and effective diameters of 12 and 10 mμ, respectively, but with the actual shapes unknown.

An interesting phenomenon shown by viruses having a very asymmetric shape and apparently directly dependent upon a rod-like shape, is the formation of two distinct layers when rather concentrated solutions of such viruses are allowed to stand (5). The line of demarcation is very sharp and gradually rises with time. The upper layer is the more dilute and shows double refraction only when caused to flow, whereas the lower layer is the more concentrated and is spontaneously doubly refracting. The lower layer appears to result when the rod-shaped virus molecules become sufficiently concentrated so that they lose their ability to rotate about their two shorter axes, and appears to consist, therefore, of a 3-dimensional mosaic of regions arranged at random to each other but in each of which all of the molecules lie approximately parallel. The phenomenon appears to be quite analogous to that first reported by Zocher and Jacobsohn (76) for vanadium pentoxide sols. Lauffer (77) concluded that the lower layer represents a special case of double refraction because it has no extinction direction.

He found the double refraction exhibited by tobacco mosaic virus to be due largely, if not entirely, to the shape of the particles and scarcely, if at all, to intrinsic double refraction. The layering phenomenon as well as the variation of the intermolecular x-ray pattern with concentration of virus is also of interest in connection with the long range electrical forces which have been the subject of several recent theoretical papers (77a, 77b). It has been suggested that these electrical forces could be effective over unusually long distances and the results with tobacco mosaic virus provide experimental evidence for the existence of forces effective between molecules over distances of a hundred or more Å. The reproduction of viruses as well as the duplication of cells may be dependent upon such forces.

Bawden and Pirie (5) found that the purification of tobacco mosaic virus by chemical means resulted in a loss in activity and filterability and an increase in stream double refraction and concluded that the purification had caused an aggregation of the virus. Similar results have been reported in the cases of other viruses, and as a result there has been a tendency on the part of virus workers to assume that purification as such must invariably cause aggregation of virus and hence that it is impossible to isolate virus in an unaltered condition. The question of the aggregation of tobacco mosaic virus was studied by the writer with Loring and Lauffer (26), and it was found that virus purified by rapid chemical treatment in the cold, or preferably by means of ultracentrifugation in the cold, was entirely comparable to the virus in untreated juice with respect to specific activity, filterability, and stream double refraction. However, merely allowing such purified virus to stand for a short time in the presence of low concentrations of salt at room temperature was sufficient to cause aggregation. Similar results have been obtained with latent mosaic and tobacco ring spot viruses. It should be recognized, therefore, that, although viruses may be aggregated readily by chemical treatment and most of the chemically purified virus preparations that have been isolated have consisted of aggregated virus, it is possible nevertheless to isolate tobacco mosaic virus in a state and possessing a specific activity, filterability, and stream double refraction comparable to that of virus in unpurified preparations.

The occasional suggestions that, in untreated juice, tobacco mosaic virus consists of small units or that active low molecular weight material may be produced following a given treatment are not in accord with results obtained in the writer's laboratory. If active low molecular weight material existed, it would not be possible to sediment the active

units in a centrifuge at a speed at which other low molecular weight material, such as hemoglobin, fails to sediment. However, it is an experimental fact that, when untreated juice or preparations supposed to contain low molecular weight active material are mixed with hemoglobin and centrifuged at a speed which will sediment ordinary tobacco mosaic virus but not hemoglobin, the virus activity is found in the sediment and not with the unsedimented hemoglobin in the supernatant liquid. This is good evidence that virus in untreated juice does not consist of low molecular weight material. Similar results have been obtained by Hughes, Pickels, and Horsfall (78) in a study on the differential centrifugation of proteins. These workers demonstrated that hemocyanin could be separated from egg albumin and that yellow fever virus could be separated from serum protein without any difficulty by means of differential centrifugation. It may be concluded that to date there has been no convincing demonstration of the existence or production of low molecular weight material carrying virus activity.

Nature and mode of action of viruses. The isolation of several of the viruses in the form of high molecular weight proteins and the demonstration, beyond a reasonable doubt, that the preparations are essentially pure and that virus activity is a specific property of the proteins necessitate a consideration of virus activity in terms of these materials. The viruses vary in composition from the amino and nucleic acids of tobacco mosaic and tobacco ring spot viruses to the amino acids, nucleic acid, carbohydrate, and lipoid that go to make up vaccine virus. Despite the variation in composition and size, there is no reason to believe that viruses differ among themselves in any fundamental respect, or that there is other than a continuity of structure from small to large viruses. All viruses have in common the ability to reproduce or multiply when placed within susceptible living cells and, since no virus has been found to multiply under any other conditions, they may be considered obligate parasites. The manner in which viruses multiply has been, and remains, a matter of much conjecture. In the case of phage, Northrop (12) considers that multiplication may be more simply explained by analogy with the autocatalytic formation of pepsin and trypsin than by analogy with the far more complicated system of living organisms. This seems a reasonable attitude, especially in view of Krueger's (79, 80) evidence for the existence of an inactive precursor or pro-phage. However, many difficulties become apparent when this viewpoint is adopted for viruses in general, for no evidence has been obtained for the existence of inactive precursors having chemical properties similar

to those of the viruses, no virus has been produced *de novo* or in the absence of living cells, and a multiplicity of precursors must be postulated, since a given host cell is capable of producing any one of hundreds of different viruses or virus strains.

Nevertheless, it is quite possible that the basic idea of catalysis may be correct with respect to virus activity. Bergmann (81) has shown experimentally that a catalyst, a protein enzyme, can cause the formation or synthesis of a peptide linkage. There is every reason to believe that proteins are produced through the formation of peptide linkages, hence it is but a step to consider that proteins may result from the catalytic action of still other proteins, and but another step to consider that a protein may catalyze reactions resulting in the formation of replicas of itself. The latter protein, catalyzing such reactions within a cell, would conform, of course, to our definition of a virus. The virus reaction may, therefore, resemble the pepsin and trypsin activation reactions and the reaction postulated for phage, except that the virus reaction is far more complicated, requiring not one but a series of reactions and special conditions which so far have not been reproduced outside of a cell. However, the great advances that have been made in recent years with respect to our knowledge of enzymes, hormones, organizers, and genes, and the work of Keilin (82), Warburg (83), Green (84), and others on intracellular reactions lead one to suspect that elucidation of the virus reaction is not beyond our grasp.

The introduction of a virus most certainly diverts the normal metabolic activity of a cell, yet the influence of the virus might be likened to that of agents already present which direct normal metabolism except that the virus exerts a dominating influence. The mechanism by means of which virus is synthesized within a diseased cell must be very similar to that by means of which normal proteins and constituents are synthesized within a normal cell. Levaditi (59) has postulated the existence of two factors within cells, a "constructive" and a "lytic" factor, and supposes that in the case of a virus-infected cell the "constructive" factor of the virus superimposes itself and dominates the normal factor. Similar ideas have been advanced in the writings of other workers, especially when cancerous cells were under consideration (85-87). In some instances the loss, rather than the addition, of a factor has been postulated. One great point of difference is that in the case of the virus reaction the "key" to the disrupted metabolism, that is to say, the virus, can be separated, isolated in pure form, and studied apart from the system, whereas in the cases of normal cells or of cancerous cells

similar "keys" have not been found separable as yet. The "key or keys" must be contained within the chromosome and probably are represented by genes or gene derivatives, hence they may be nucleoproteins. The fact that all viruses so far isolated have been found to consist of or to contain nucleoprotein may be of special significance, and the possibility that they may have been derived from genes or nuclear material has been considered from time to time by different writers (88–92). Although it is quite possible that viruses may have arisen originally in such an endogenous manner, it should be emphasized that, as recognized today, virus activity results only from the introduction of virus from without. There is, however, a strong and growing tendency to consider that viruses or similar factors may, upon provocation of the cell, originate endogenously and give rise to tumors or cancers (93–95). Whether such viruses are actually derived from normal cell constituents or are formed by the mutation of a "masked" virus normally or usually carried within the cell, but in reality alien to the cell, is not known. The probability that the synthesis of viruses does not differ fundamentally from the synthesis of normal proteins within cells and the fact that, although viruses may be removed and studied apart, they nevertheless possess most of the properties of living organisms, cause the attack on the nature and mode of action of viruses to become of signal importance, for it is in reality an attack not only on the problems of abnormal and normal metabolism, but also on the nature of life. The difficulties, discussed by Bohr (96), involved in making studies of the living state without affecting conditions by the very act of study, are lessened in the case of viruses, since they may be removed from cells, subjected to various studies, and then reintroduced into cells without measurably affecting them. This property of suspended animation is unique with viruses and is possessed by no other entity to a similar degree, for the same difference that exists between viruses and ordinary living organisms must exist between viruses and the seeds or spores that are usually cited as examples of suspended animation. This and other preceding statements are necessarily limited by one's understanding of the nature of the difference between viruses and living organisms, hence this question will be considered.

The chemist, after a perusal of the properties of the purified viruses that have been isolated and adequately studied, has no difficulty in coming to the conclusion that they are protein molecules. The chemical and physical properties are admittedly those to be expected of large protein molecules, and the chemist, well acquainted with the catalytic

synthesis of a peptide bond and the autocatalytic formation *in vitro* of the pepsin and trypsin proteins, views virus activity as but a logical and not unexpected extension of the expression of chemical structure. True, the duplication of conditions necessary for the expression of virus activity *in vitro* has not yet been accomplished and but little has been done with the large and complex viruses such as vaccine virus, but the chemist, secure in the knowledge already gained, considers that such problems lend themselves to experimentation and looks forward with confidence to their eventual solution.

The pathologist and biologist, long interested in the expression of virus activity and its results, have, on the other hand, considered viruses to be small living organisms. Recently, however, there has been a tendency on the part of some workers to accept the view that some or all viruses may be something other than living organisms. Rivers (97) has suggested that the large viruses may be small living organisms, the middle-sized viruses representatives of an unknown form of life, and the small viruses non-living agents, but that it is impossible to draw lines dividing the groups and that very probably one group shades off into its neighbors. Rivers appears to believe, therefore, that, although the large differ from the small viruses, they nevertheless form a continuum from small non-living to large living viruses. Green (98) and others have suggested that viruses are simplified fragments of living protoplasm arising from organisms by a process of retrograde evolution under parasitism involving loss of function and associated substance, and that this process may result in forms varying from a single colloidal molecule to entities almost indistinguishable from ordinary living organisms. Laidlaw (99) has presented a similar view and has assembled much evidence in its support. Although he rejects the simple "autocatalytic protein" view of viruses, he supposes that viruses arise by a gradual loss of substance and of such functions as enzyme systems. He offers no alternative theory of the mechanism of virus action and appears to consider that viruses are formed much as normal cellular constituents are formed. As mentioned above, this view, because of the recent work on intracellular reactions, must have catalysis as its basis. The general situation with respect to bacteriophage has been quite comparable, for d'Hérelle (100) first postulated that phages were sub-microscopic living entities, probably constituted of simple colloidal micellae, and later Bordet (90) considered them to be non-living transmissible lytic principles. Wollman (91) regarded phages as being derived from the bacteria and discussed the possibility of their being

similar in nature to a gene. Doerr (101) has just published a masterly discussion of the nature of viruses and bacteriophages in which he has presented and evaluated the ideas held by different workers. He points out that the large viruses are similar to the small viruses in all important respects, that there is no reason for attempting to separate them on the basis of size, and that, furthermore, no fundamental difference between viruses and living organisms has been demonstrated. In Doerr's opinion, the chief point of issue is whether non-living infectious agents should be accepted along with living infectious entities or the smallest virus accepted as a living agent, and after a thorough discussion he tends to resolve the conflict in favor of the acceptance of viruses, even though they may consist of a protein molecule, as living. The pathologist has been able to rationalize his knowledge of viruses with his knowledge of living organisms so that he is willing to accept the smallest virus, even though a large nucleoprotein molecule, and the largest living organism as representatives of a common series in which there is a definite but almost imperceptible gradation of substance and of complexity of function.

We come, therefore, to the interesting situation where the pathologist and the chemist have a common meeting ground, a territory never before accessible with certainty. Needham (102) has argued the necessity of a bridge between morphology and biochemistry, and the essence of this bridge now appears to have been achieved between pathology and chemistry. The chemist, always interested in atoms and molecules, has recently extended his knowledge in two directions. At one extreme, nuclear chemistry has achieved the transmutation of elements and at the present time is pushing vigorously ahead with studies on the manner in which the protons and electrons that make up the atoms are arranged and on ways and means of breaking up large and complex structures into simpler ones. Instead of being homogeneous, the atoms appear to consist of a virtual maze of discontinuities, but nevertheless in perfect order as shown by the breakdown of the large uranium atom into a barium atom. At the other extreme, the chemist has found the virus proteins, molecules larger than those ever known before. However, the chemist realizes that just as the chemical, biological, and physical properties of ordinary molecules are a direct result of their structure, so too must the properties of atoms and of the viruses be a direct result of their structure. This structure, whether evidenced by the proton and electron of the hydrogen atom, by the atoms of the water molecule, or by the units combined to make up a

virus, must be fundamentally the same. The recognition of the essential identity of the structure of entities, regardless of their nature, that is, of structure as a continuum from smallest to largest, is of fundamental importance. Although recognizing the significance and importance of structure, many have been led astray because of attempts to separate the living from the non-living on the basis of certain characteristics, not realizing that, as Pirie has so well stated in his essay on the subject, the word "life" is merely a definition of degree. Aristotle is credited with saying, over two thousand years ago, that "Nature makes so gradual a transition from the inanimate to the animate kingdom that the boundary lines which separate them are indistinct and doubtful." Attempts to arrive at a definite line of division have resulted in failure in the past and appear doomed to a similar fate in the future. Fortunately, the classification of an entity as living or non-living, or as a cell or a molecule, is of little or no importance, whereas the complete realization of the expression inherent in structure is of tremendous importance.

It has been stated before (103) that from the standpoint of structure there is no reason why a single structural entity, which we call a molecule, should not be larger than the ordered group of structural entities which we call a cell. The overlapping of structure in the case of atoms is well known, and the recent demonstration of the formation of barium and other atoms from a single uranium atom brings it even more forcibly to attention. A similar situation may prevail at the other extreme of size. Neither the cell nor the atomic theory should be handicapped by a reference to the living state, but should be utilized only to define certain accepted orders of structure. It should also be recognized that there may be such a gradual transition between these accepted orders of structure that the designation of intervening entities as molecules or as cells becomes one merely of personal preference. The nature of the bonds between units within cells, as well as the nature of the bonds which hold together the large nucleoproteins, requires much more investigation before they may be completely understood. However, at the present time there is no reason to believe that they differ in any fundamental respect from the forces already known to exist in atoms. It becomes possible, therefore, to blend the atomic theory, the germ theory, and the cell theory into a unified philosophy, the essence of which is structure or architecture. The chemical, biological, and physical properties of matter, whether atoms, molecules, germs, or cells, are directly dependent upon the structure of the matter, and the results of

the work with viruses have permitted the conclusion that this structure is fundamentally the same regardless of its occurrence.

REFERENCES[1]

(1) IWANOWSKI, D. Bull. Acad. Imp. Sci. St. Petersburg, n.s. **35:** 67, 1892.
(2) BEIJERINCK, M. W. Verh. Akad. Wetensch. Amsterdam II, no. 5, **6:** 1, 1898.
(3) STANLEY, W. M. Science **81:** 644, 1935; Phytopath. **26:** 305, 1936.
(4) STANLEY, W. M. J. Bact. **31:** 52, 1936; J. Biol. Chem. **117:** 325, 1937.
(5) BAWDEN, F. C. AND N. W. PIRIE. Proc. Roy. Soc. B **123:** 274, 1937.
(6) STANLEY, W. M. AND R. W. G. WYCKOFF. Science **85:** 181, 1937.
(7) LORING, H. S. AND R. W. G. WYCKOFF. J. Biol. Chem. **121:** 225, 1937.
(8) BAWDEN, F. C. AND N. W. PIRIE. Brit. J. Exper. Path. **19:** 66, 1938.
(9) BEARD, J. W. AND R. W. G. WYCKOFF. Science **85:** 201, 1937.
(10) BAWDEN, F. C. AND N. W. PIRIE. Brit. J. Exper. Path. **19:** 251, 1938.
(11) BAWDEN, F. C. AND N. W. PIRIE. Brit. J. Exper. Path. **18:** 275, 1937.
(11a) PIRIE, N. W., K. M. SMITH, E. T. C. SPOONER AND W. D. MCCLEMENT. Parasitol. **30:** 543, 1938.
(11b) PRICE, W. C. AND R. W. G. WYCKOFF. Phytopath. **29:** 83, 1939.
(12) NORTHROP, J. H. Science **84:** 90, 1936; J. Gen. Physiol. **21:** 335, 1938.
(13) CLAUDE, A. Science **87:** 467, 1938.
(14) WYCKOFF, R. W. G. Proc. Soc. Exper. Biol. and Med. **36:** 771, 1937.
(15) JANSSEN, L. W. Ztschr. Hyg. **119:** 558, 1937.
(16) CLAUDE, A. Proc. Soc. Exper. Biol. and Med. **39:** 398, 1938.
(17) STANLEY, W. M. Science **83:** 626, 1936.
(18) WYCKOFF, R. W. G., J. BISCOE AND W. M. STANLEY. J. Biol. Chem. **117:** 57, 1937.
(19) CALMETTE, A. AND C. GUÉRIN. Ann. Inst. Pasteur **15:** 161, 1901.
(20) HOLMES, F. O. Bot. Gaz. **87:** 39, 1929.
(21) SAMUEL, G. AND J. G. BALD. Ann. Appl. Biol. **20:** 70, 1933.
(22) YOUDEN, W. J. AND H. P. BEALE. Contr. Boyce Thompson Inst. **6:** 437, 1934.
(23) LORING, H. S. J. Biol. Chem. **121:** 637, 1937.
(24) ROUS, P., J. G. KIDD AND J. W. BEARD. J. Exper. Med. **64:** 385, 1936.
(25) PARKER, R. F. AND T. M. RIVERS. J. Exper. Med. **64:** 439, 1936; **67:** 725, 1938.
(26) LORING, H. S., M. A. LAUFFER AND W. M. STANLEY. Nature **142:** 841, 1938.
(27) LORING, H. S. J. Biol. Chem. **126:** 455, 1938.
(28) HOLMES, F. O. Phytopath. **28:** 58, 1938.
(29) CHESTER, K. S. Phytopath. **26:** 715, 1936.
(30) JENSEN, J. H. Phytopath. **23:** 964, 1933; **26:** 266, 1936; **27:** 69, 1937.
(31) MCKINNEY, H. H. J. Agric. Res. **51:** 951, 1935.
(32) KUNKEL, L. O. Phytopath. **24:** 13, 1934.

---

[1] For more complete bibliographies, see W. M. STANLEY, Recent advances in the study of viruses, in Science in Progress, Yale University Press, New Haven, 1939, and Biochemistry and biophysics of viruses, in Handbuch der Virusforschung, Springer, Wien, 1938.

(33) Stanley, W. M. Harvey Lectures 33: 170, 1938; also in Bull. N. Y. Acad. Med. 14: 398, 1938.
(34) Merrill, M. H. J. Immunol. 30: 169, 1936.
(35) Ross, A. F. and W. M. Stanley. J. Gen. Physiol. 22: 165, 1938.
(36) Frampton, V. L. and A. M. Saum. Science 89: 84, 1939.
(37) Stanley, W. M. and M. A. Lauffer. Science 89: 345, 1939.
(38) Sreenivasaya, M. and N. W. Pirie. Biochem. J. 32: 1707, 1938.
(39) Stanley, W. M. J. Biol. Chem. 117: 755, 1937.
(40) Gratia, A. and P. Manil. Compt. Rend. Soc. Biol. 126: 903, 1937.
(41) Neurath, H. and A. M. Saum. J. Biol. Chem. 126: 435, 1938.
(42) Hills, C. H. and C. G. Vinson. Missouri Agric. Expt. Sta. Res. Bull. 286: 1938.
(43) Seastone, C. V., H. S. Loring and K. S. Chester. J. Immunol. 33: 407, 1937.
(44) Eriksson-Quensel, I.-B. and T. Svedberg. J. Am. Chem. Soc. 58: 1863, 1936.
(45) Wyckoff, R. W. G. J. Biol. Chem. 121: 219, 1937; 122: 239, 1937–38.
(46) Lavin, G. I. and W. M. Stanley. J. Biol. Chem. 118: 269, 1937.
(47) Hollaender, A. and B. M. Duggar. Proc. Nat. Acad. Sci. 22: 19, 1936.
(48) MacFarlane, M. G. and M. H. Salaman. Brit. J. Exper. Path. 19: 184, 1938.
(48a) Rischkov, V. L. and K. S. Soukhov. Compt. Rend. (Doklady) URSS 21: 265, 1938.
(49) Ross, A. F. and W. M. Stanley. J. Biol. Chem. 128: lxxxiv, 1939.
(49a) Kögel, F. and H. Erxleben. Ztschr. f. physiol. Chem. 258: 57, 1939.
(50) Ross, A. F. and W. M. Stanley. J. Am. Chem. Soc. 61: 535, 1939.
(50a) Greenstein, J. P. J. Biol. Chem. 128: 233, 1939.
(51) Loring, H. S. J. Biol. Chem. 123: 126, 1938; 128: lxi, 1939.
(52) Mirsky, A. E. and L. Pauling. Proc. Nat. Acad. Sci. 22: 439, 1936.
(53) Bernal, J. D. and I. Fankuchen. Nature 139: 923, 1937.
(54) Astbury, W. T. Cold Spring Harbor Symposia 6: 359, 1939.
(55) Bernal, J. D. Proc. Roy. Soc., B 125: 299, 1938.
(56) Steinhardt, J. J. Biol. Chem. 123: 543, 1938.
(57) Smith, J. H. Ann. Appl. Biol. 25: 227, 1938.
(58) Pirie, N. W. *In:* Perspectives in biochemistry. Cambridge Univ. Press, London, 1938.
(59) Levaditi, C. La Presse Médicale 102: 1889, 1938.
(60) Wyckoff, R. W. G. and R. B. Corey. J. Biol. Chem. 116: 51, 1936.
(61) Kausche, G. A. Naturwissensch. 27: 77, 1939.
(62) Iwanowski, D. Ztschr. Pflanzenkr. 13: 1, 1903.
(63) Beale, H. P. Contr. Boyce Thompson Inst. 8: 413, 1937.
(64) Best, R. J. Nature 139: 628, 1937; 140: 547, 1937.
(65) Bernal, J. D. Proc. Roy. Soc. Med. 31: 208, 1937.
(66) Bawden, F. C. Proc. Roy. Soc., B 125: 297, 1938.
(67) Elford, W. J. See p. 126 *in:* Handbuch der Virusforschung. Springer, Wien, 1938.
(68) Svedberg, T. Chem. Rev. 20: 81, 1937; Nature 139: 1051, 1937.
(69) Lauffer, M. A. and W. M. Stanley. J. Biol. Chem. 123: 507, 1938.

(70) TAKAHASHI, W. N. AND T. E. RAWLINS. Proc. Soc. Exper. Biol. and Med. **30**: 155, 1932.
(71) FREUNDLICH, H. *In:* Colloid and capillary chemistry. Methuen & Co., London, 1926.
(72) LAUFFER, M. A. Science **87**: 469, 1938; J. Biol. Chem. **126**: 443, 1938.
(72a) KAUSCHE, G. A., E. PFANKUCH AND H. RUSKA. Naturwissensch. **27**: 292, 1939.
(73) LAUFFER, M. A. AND W. M. STANLEY. Chem. Rev. **24**: 303, 1939.
(74) LANGMUIR, I. AND V. SCHAEFER. J. Am. Chem. Soc. **59**: 1406, 1937.
(75) SMITH, K. M. AND W. D. MACCLEMENT. Proc. Roy. Soc., B **125**: 295, 1938.
(76) ZOCHER, H. AND K. JACOBSOHN. Kolloid Ztschr. **41**: 220, 1927.
(77) LAUFFER, M. A. J. Phys. Chem. **42**: 935, 1938.
(77a) LANGMUIR, I. J. Chem. Physics **6**: 873, 1938.
(77b) LEVINE, S. Proc. Roy. Soc. A, **170**: 145, 1939.
(78) HUGHES, T. P., E. G. PICKELS AND F. L. HORSFALL, JR. J. Exper. Med. **67**: 941, 1938.
(79) KRUEGER, A. P. AND J. FONG. J. Gen. Physiol. **21**: 137, 1937.
(80) KRUEGER, A. P. AND J. H. MUNDELL. Science **88**: 550, 1938.
(81) BERGMANN, M. AND C. NIEMANN. Science **86**: 187, 1937.
(82) KEILIN, D. Proc. Roy. Soc., B **98**: 312, 1925; **104**: 206, 1929.
(83) WARBURG, O. AND W. CHRISTIAN. Biochem. Ztschr. **266**: 377, 1933.
(84) GREEN, D. E. Biochem. J. **30**: 629, 1936.
(85) CLAUDE, A. AND J. B. MURPHY. Physiol. Rev. **13**: 246, 1933.
(86) MURPHY, J. B. Bull. Johns Hopkins Hosp. **56**: 1, 1935.
(87) GREEN, R. G. Biodynamica **39**: 1, 1938.
(88) DUGGAR, B. M. AND J. K. ARMSTRONG. Ann. Mo. Bot. Gard. **10**: 191, 1923.
(89) BAIL, O. Deutsch. Med. Wchnschr. **15**: 13, 1925.
(90) BORDET, J. Proc. Roy. Soc. **107**: 398, 1931.
(91) WOLLMAN, E. Bull. Inst. Pasteur **35**: 1, 1937; Ann. Inst. Pasteur **60**: 13, 1938.
(92) LINDEGREN, C. C. J. Heredity **29**: 409, 1938.
(93) ROUS, P. AND J. W. BEARD. J. Exper. Med. **62**: 523, 1935.
(94) ROUS, P., J. W. BEARD AND J. G. KIDD. J. Exper. Med. **64**: 401, 1936.
(95) ROUS, P. The Harvey Lectures. P. 74, Williams & Wilkins Co., Baltimore, 1936.
(96) BOHR, N. Nature **131**: 421, 1933.
(97) RIVERS, T. M. Physiol. Rev. **12**: 423, 1932; Bull. N. Y. Acad. Med. **14**: 383, 1938; Lane Lectures, Stanford University, 1939.
(98) GREEN, R. G. Science **82**: 443, 1935.
(99) LAIDLAW, P. P. Rede Lecture, Cambridge University Press, London, 1938.
(100) D'HÉRELLE, F. The bacteriophage and its behavior. Williams & Wilkins Co., Baltimore, 1926.
(101) DOERR, R. Chapter I, Handbuch der Virusforschung. Springer, Wien, 1938.
(102) NEEDHAM, J. Order and life. Cambridge University Press, London, 1936.
(103) STANLEY, W. M. Am. Nat. **72**: 110, 1938.

FAT TRANSPORT IN THE ANIMAL BODY

W. R. BLOOR

Department of Biochemistry and Pharmacology, School of Medicine and Dentistry, The University of Rochester, Rochester, New York

The problem of fat transport differs essentially from that of protein or carbohydrate transport in that the fatty acids, the elemental constituents of the fat, are insoluble in water as are also most of their compounds, while the corresponding elements of protein and carbohydrate are readily water-soluble. The digestion products of the proteins and carbohydrates enter the blood directly and are carried as such, while the fats are resynthesized during absorption, are transported in the lymph, and are discharged into the blood and distributed mainly as fat in a fine state of division. The mechanisms provided in the animal body for the carriage of the fatty acids and their compounds in the blood and across the various cellular barriers are consequently quite different from those provided for the other foodstuffs. Conceptions of the method of transport have changed considerably in the fifteen years since the last review was written. Important new tools for use in the study of fat transport have been found in the tagged or labelled fatty acids which it is now possible to trace on their way through the organism, and in radioactive phosphorus, used first by Hevesy (1935) and later by many workers including Perlman and Chaikoff (1939a), Artom, Sarzana and Segré (1938), Chargaff (1939) and Haven and Bale (1939), which combines with the fatty acids in the lecithin type of combination. The marked fatty acids used are: 1, the iodized acids as used by Artom (1933a); 2, highly unsaturated acids as used by Sinclair (1929); 3, elaidic acid by Sinclair (1935b) and Kohl (1938); 4, acids containing deuterium (Schoenheimer and Rittenberg, 1935; Barrett, Best and Ridout, 1938; Cavanagh and Raper, 1939); and 5, fatty acids with characteristic absorption spectra such as eleostearic acid and activated linoleic acid by Burr and associates (Miller, Barnes, Kass and Burr, 1939). It is proposed in the present review to sketch briefly the new facts and ideas in their bearing on the problem.

Transfer across the intestinal mucous membrane—Fat absorption. It is well recognized that the bile and the lipases of the

pancreas and intestine are important parts of the mechanism for the transfer of fat from the intestine to the blood. Work in the last few years has provided a fresh insight into the mechanism of their activity. In addition, the phosphorylation of the fats—the formation of the more mobile and perhaps more reactive phospholipids—has introduced a new factor which is perhaps more important than the known ones with respect to fat transport.

Fat splitting. As was concluded in the previous review of this topic, practically all evidence is in favor of a complete splitting of the fat during its stay in the normal intestine. Even in those cases in which there is little absorption, it is the exception to find unsplit fat in the feces. For that reason it can be assumed that the first stage in the transport of fat from the intestine is complete splitting into its constituent fatty acids and glycerol. The same holds for other hydrolyzable fatty acid compounds, the phospholipids, lecithin and cephalin. The difficultly hydrolyzable fatty acid compounds, as far as they have been investigated, such as sphingomyelin, cerebrosides, and cholesterol esters, are not absorbed.

Soap formation. Much has been made of the fact that the intestine is slightly acid (pH 6.7) throughout most of its length, from which it was concluded that there could be little if any soaps present and there could then be little absorption of the fatty acids in the form of the water-soluble soaps. On the other hand, the compounds of the fatty acids with bile salts are stable and diffusible down to a pH of 6.2 and these compounds rather than the soaps are now regarded as the form of absorption of the fatty acids. Soaps are probably essential in the primary emulsification of fat required for its rapid hydrolysis by the lipase. The lipases being water-soluble have contact with the fat only at its surface. Hence, it is desirable that the surface be made as great as possible, which is accomplished by the emulsification. After the lipase has made contact with the fine fat particles, it is probable that splitting is rapid and a continued state of emulsification is not necessary. Since both the pancreatic juice and bile are alkaline at the time of entry into the intestine, conditions at that time are right for soap formation by combination of the secreted alkali with the free fatty acid always present in the food fat. Emulsification of the unsplit fat could then take place and probably persist long enough for hydrolysis to take place.

The train of events in the absorption of fat from the intestine may then be outlined as follows. The fat of the food, which in ordinary diets does not constitute more than 15 per cent of the dry weight of the

food, always contains a small percentage (less than 10 per cent) of free fatty acid. This amount is increased to a variable extent during its stay in the stomach, due in part to the gastric lipase. Fat in the food slows the emptying of the stomach so that the fat is delivered to the intestine in small portions. In the duodenum, it meets the alkaline secretions of the pancreas and liver, and the free fatty acid is neutralized forming soap which rapidly emulsifies the unsplit fat. At the same time the emulsified fat is mixed with the pancreatic lipase and digestion proceeds rapidly. The fatty acids and soaps formed by the digestion combine with the bile salts and are absorbed with their help. The removal of the split-products by absorption speeds up the hydrolysis of the unsplit fat. Making use of the labelled fat elaidin, Kohl (1938) showed that, after an initial lag, fat is absorbed from the intestine of rats at a nearly constant rate.

Whether any unsplit fat is absorbed as such, swept in with the current of split-products or by direct penetration, cannot be stated, but from present evidence as regards normal intestinal behavior it seems unlikely. Evidence of direct absorption of fat without hydrolysis under certain conditions continues to appear. Kitagawa (1934) reported that finely dispersed fat was directly absorbed without preliminary hydrolysis from the large intestine and the lower part of the small intestine of dogs. Onozaki (1936) obtained similar results. The absorption of even the very finely divided fat was very slow—from $\frac{1}{6}$ to $\frac{1}{16}$ as fast as absorption after splitting. Doubilet and Reiner (1937), taking advantage of an accidental Thiry-Vella fistula in the middle ileum in a human being, found that oleic acid and olive oil were readily absorbed in the absence of bile acids and that there was but little improvement when bile acids were used. On the other hand, Riegel, Elsom and Ravdin (1935) found that while the absorption of oleic acid from jejunal loops in dogs was slight, the presence of sodium taurocholate and to a less extent hepatic or gall bladder bile greatly increased the absorption.

Different parts of the intestine probably behave differently as regards the absorption of unsplit fat. Also the possibility of splitting by lipases in the intestinal juice has not been excluded.

Bile. The importance of bile in fat digestion and absorption has been recognized for a long time and it is probably the greatest single factor in the transport of fat from the intestinal lumen into the absorbing cells. It is more important than the pancreatic lipase since the functions of this secretion can be taken over by other lipases in the intestine.

Work which throws light on the part taken by the bile was supplied

by Verzár and Kúthy (1929) who found that sodium salts of glycocholic and taurocholic acids dissolve palmitic and stearic acids and that the combination is diffusible and therefore molecularly dispersed. The solution was found to be stable in buffers at pH 6.2, i.e., at an acidity greater than prevails in the small intestine. These findings offer an explanation of the effect of bile on fat absorption and also dispose of the difficulty raised by the acidity of the intestinal contents which would make difficult or impossible the preservation of a soap emulsion in the intestine. Verzár and Kúthy thought at first that the association products of bile salts and fatty acids, i.e., compounds containing as much as 8 molecules of bile salt to 1 of fatty acid were absorbed as such. Calculation showed them that this assumption would require an amount of bile salt much greater than could be supplied by the normal secretion of bile and would require either a circulation of bile salts or some other mechanism of absorption. The hypothesis finally offered was that the bile salts become fixed in the intestinal mucous membrane and by their solvent effect bring about the passage of the fatty acids into the epithelial cells. The finding of Breusch (1937) that saturated fatty acids from C_{16} upwards and also oleic acid in presence of bile salts were not diffusible through parchment was explained by Verzár as due to the low permeability of Breusch's membranes. Breusch found further that the saturated acids below C_{16} down to C_{10} were increasingly diffusible while the solubility in bile salts decreased with increasing molecular weight. Of the acids of lower molecular weight, the soaps were often much more diffusible than the bile salt. These experiments were made with parchment membranes and the behavior of such membranes may be quite different from that of the living intestinal epithelium. Other authors (Szörényi, 1932; Müller, 1932) confirm Verzár and Kúthy in finding that the higher fatty acid compounds are diffusible. Whether or not lipid material can be moved across membranes seems to depend on its chemical nature, probably on its ability to combine with substances in the membranes which act as carriers, e.g., protein, bile salts, etc. Breusch found a quite sharp specificity in absorption of material from the fat stores. Fat and cholesterol moved readily while paraffin did not. Cholesterol was absorbed from the intestine while coprosterol was not.

Absorption into the portal circulation. Water-soluble metabolites absorbed from the intestine are collected by the portal system and carried to the liver. Relatively little is absorbed by the lymph system. The water-insoluble fats, by contrast, are largely absorbed via the lymph system and reach the blood indirectly. It is still a disputed

point whether any appreciable amount of fat is absorbed directly into the blood. The observation which, more than any other, keeps alive the dispute is the old one of Munk and Rosenstein who were able to recover only 60 per cent of the absorbed fat by a chyle fistula. It was not certain in their subject that the fistula delivered all the chyle; some may have passed into the blood by other channels, so that there is a good possibility that there may not have been any appreciable direct absorption. Nevertheless, the missing 40 per cent has stimulated efforts to account for it mainly by demonstrating an increase in the fat of the portal system over that in the general circulation, which would indicate a direct absorption into the blood. Water-soluble compounds of the fatty acids, such as the soaps of the short-chain fatty acids or of such oxidized fatty acids as are believed by Tangl and Berend (1930) to be formed during fat digestion would be expected to appear in the portal system, but in the digestion of ordinary fat it has not been demonstrated that such compounds are formed.

Brockett, Spiers and Himwich (1934) found no change in the lipid content of serum of portal blood during absorption of oil, which is in agreement with the results of most preceding work. On the other hand, Cantoni (1928) found that the portal blood contained definitely more fatty acid and less cholesterol than the carotid blood. The absorption of fat directly into the blood in the form of phospholipid is a possibility which has not yet been investigated. Eckstein (1925), after diverting the lymph of the thoracic duct by fistula, found a small but appreciable increase in the total fatty acids of the blood during fat absorption while there was no change in the blood of the controls not fed fat. The situation as regards absorption of fat directly into the blood remains unsettled.

Histological investigations of fat absorption. Rossi (1936), confirming his earlier work, found that the intestinal mucosa on immersion in a solution of fatty acids in bile salts, even when fixed with formaldehyde, could take up the fatty acids which were demonstrable as fine granules inside the cells. Neutral fat dissolved in bile acids did not diffuse. Free fatty acids alone diffused but much more slowly. Treatment of the formalin-fixed intestine with fat solvents destroyed its power to take up the fatty acids, which led to the conclusion that the lipid of the absorbing cells was the effective agent in the absorption. Jeker (1936) and Verzár and Jeker (1936) reported experiments which appeared to show the rapid absorption of fatty acid from the intestine and its later change into fat. For the demonstration of fatty acids, Fischler's stain

was used but in view of the criticism of Fischler's stain by Kaufmann and Lehmann (1926) which Jeker and Verzár did not attempt to meet, their conclusions are doubtful. On the other hand, the photographs of the sections make it certain that some material, presumably fatty acid, appears in the epithelial cells within a few minutes after exposure to the fatty acid-bile mixture and that the absorbed material undergoes a change which makes it no longer stainable with Fischler's stain but stainable with Sudan. The change was prevented by iodoacetate and by adrenalectomy and restored by injections of adrenal cortical hormone.

THE LIVER AND FAT TRANSPORT. That the liver takes an active part in fat transport is generally accepted. The old observation of a mobilization of fat to the liver in starvation and under the action of various drugs was interpreted as a step in the preparation of fat for metabolic use. The liver also acts as a temporary storage place for fat when it is being absorbed from the intestine just as it acts as a temporary store for carbohydrate and protein products. The nature of the changes brought about in the fat by the liver during the period of storage is becoming apparent from recent work and appears to be essentially phosphorylation, a change into lecithin, which was the assumption made on purely theoretical grounds by the earlier workers. Moreover, it is becoming increasingly apparent that the phosphorylated product is actually lecithin and not other phospholipids. The fat content of the liver does not change much in ordinary normal metabolism. The fat which comes there is worked over promptly and moved out. The phospholipid content changes even less and it is only under exceptional circumstances that there is a change in its percentage if calculated on a fat-free dry-weight basis. Even when there is a large accumulation of fat the phospholipid content if calculated on this basis remains remarkably constant (MacLachlan and Hodge 1939).

Large and "chronic" accumulations of fat are found in the liver under circumstances of which the following are the more important: 1, depancreatized dogs on certain mixed diets fed insulin (Fisher, 1924; Allan, Bowie, Macleod and Robinson, 1924; Best and Ridout, 1939). After a varying period of time there is an accumulation of fat and sometimes of cholesterol esters; 2, in high-fat, low-protein diets (Channon, Loach, Loizides, Manifold and Soliman, 1938); 3, after cystin feeding, but not after methionine (Tucker and Eckstein, 1938); 4, after cholesterol feeding (Okey and Yokela, 1936; Loizides, 1938); 5, fasting, if there is available fat in the depots; 6, anterior pituitary extract (Best and Campbell, 1938); and 7, in the hen at the onset of sexual maturity

(Lorenz, Chaikoff and Entenman, 1938). The substance which works best in removing or preventing the accumulation of fat in the liver is choline. Various other substances have been tried, e.g., derivatives or chemical relatives of choline such as betaine, creatin, choline methyl ester, tetra (β-hydroxy ethyl) ammonium chloride (Platt, 1939; Best and Ridout, 1939) and an alcoholic extract of pancreas (Dragstedt, Van Prohaska and Harms, 1936) known as lipocaic, about which findings differ as to details. Best and Ridout (1938) and MacKay and Barnes (1938a) reported that there was no active material in the lipocaic except choline and factors associated with protein, while Channon, Loach and Tristram (1938), after extensive experiments on rats, came to the conclusion that there was present a lipotropic factor in addition to choline and protein. Ralli, Rubin and Present (1938) found that if the pancreatic ducts were tied off but the pancreas left in place there was the same accumulation of fat in the liver as in depancreatized dogs. *Montgomery, Entenman and Chaikoff (1939) found also an accumulation* of fat in the livers of dogs after tying off the pancreatic duct if time enough was allowed (12–24 weeks). The formation of fatty livers was independent of weight loss and could be prevented by feeding raw pancreas. With increase of fat in the liver there was increase of cholesterol esters, but phospholipid content was not affected. Apparently something which prevents fatty livers is supplied by the pancreas, either as internal or external secretion or more likely produced by the action of the pancreatic juice on the intestine or on the food.

Anterior pituitary extract caused an increase of fat in the liver,—greater in guinea pigs and mice than in rats (Best and Campbell, 1938) which was not affected by choline or lipocaic (MacKay and Barnes, 1938b). Choline had no effect on the fatty infiltration following partial hepatectomy, an infiltration which was prevented by adrenalectomy.

The fact that choline is the most useful substance in the prevention and cure of fatty livers and the fact that colamine (aminoethyl alcohol) is not effective points to lecithin rather than cephalin as the important *metabolic intermediate in the metabolism of fat in the liver and there*fore probably in the whole organism. This idea is well supported by the evidence regarding phosphorylation of fat and by the lecithin-cephalin composition of the blood and tissues. Thus blood plasma is found to contain most of its phospholipid as lecithin (Williams, Erickson, Avrin, Bernstein and Macy, 1938; unpublished work by MacLachlan from this laboratory) while the corpuscles which take no important part in fat transport contain about 50 per cent cephalin and the rest lecithin

and sphingomyelin. In the liver the ratio is 60 lecithin to 40 other phospholipids; in muscle it is 55 lecithin to 45 cephalin; in brain 40 lecithin to 60 cephalin. Chargaff (1939) found that lecithin is synthesized in much larger amounts by the liver and intestine than cephalin. Sinclair's (1935b) hypothesis of a metabolic and a structural or functional type of phospholipid is plausible and it appears at present as though lecithin was the metabolic phospholipid with cephalin and possibly sphingomyelin as the structural or functional form. That choline actually stimulates the phospholipid turnover in the liver was shown by Perlman and Chaikoff (1939a) using the radioactive isotope of phosphorus. After choline feeding there was an increased formation as well as a rapid removal of radioactive phospholipid in the liver of rats. By the study of the effect of a single dose of choline, it was found that the increased phospholipid metabolism began about 1 hour after feeding and was over about 10 to 12 hours after. The increase in phospholipid metabolism was proportional to the amount of choline (up to 30 mgm. per rat). Cholesterol when fed is a factor in the production of fatty livers. Perlman and Chaikoff (1939b) have shown that it does so by depressing phospholipid turnover. Ingested cholesterol depressed the new formation of phospholipid (labelled). Choline fed along with cholesterol, however, gave their highest values for phospholipid turnover. These writers emphasize the fact noted for other tissues, that the phospholipid content of the liver is constant while the phospholipid turnover is not constant. Laszt and Verzár (1936a) found that agents which prevent phosphorylation such as iodoacetate and adrenalectomy prevented fatty livers after phosphorus poisoning or fasting and also prevented emptying of the fatty liver, from which they concluded that the mobilization of fat in both places was accompanied by a phosphorylation.

Blixenkrone-Møller (1938) has brought evidence to indicate that the ketone body formation by the liver, which has been known for a long time, may assume proportions which entitle it to consideration among the important mechanisms of transport of fatty acid fragments on the way to combustion. Evidence is brought to show that the liver can form these substances in large amounts but does not burn them while the muscles can burn but not create them. He has calculated that the perfused livers of depancreatized cats form up to 30 times as much ketone bodies as are excreted and that these fragments of which the ordinary fat fatty acids can yield 4 or more are consumed by the muscles. Similar conclusions were reached by Denal and associates (1936) in feed-

ing experiments with fatty acids to rats. They found that as many 4-carbon fragments, transformable into ketone body acids were supplied as the length of the fatty acid chain allowed.

Phosphorylation as a mechanism of fatty acid transport. The increase of phospholipid in the blood during fat absorption has been reported many times (Sinclair, 1937) so that its occurrence as part of the mechanism of fat transport is generally accepted. Regarding the place and time as well as the manner of its formation, considerable effective work has been done although some essential details are still missing. The suggestion of Bloor (1915) indicating its formation in the red blood corpuscles has in general not been supported and the observation of the increase of fat and to a less extent of phospholipid of the corpuscles during fat absorption is regarded as an adsorption phenomenon. Sinclair (1929) made the observation that during fat absorption the phospholipids of the intestinal mucosa changed their fatty acids to correspond with those being absorbed but did not change in amount. Verzár and Laszt (1934a, b) found that when oleic acid and bile salts were introduced into the intestine of the rat they were absorbed at a fairly constant rate which was not affected by glycerol or phosphate alone but was accelerated by glycerol and phosphate together and still more by glycerol phosphate (increase of 150 per cent). The absorption was inhibited by monoiodoacetate and phlorizin, substances known to inhibit the phosphorylation of hexoses. It was believed, therefore, that a phosphorylation of the fatty acids to phospholipid took place during fat absorption. The absorption of fat from the intestine was inhibited by the same agents and by removal of the adrenals, agents which are known to inhibit phosphorylation of the sugars. These results definitely implicate phospholipid formation in the processes of fat absorption (Verzár and Laszt, 1935). Administration of cortical extract restored normal fat absorption in adrenalectomized animals. Flavin phosphate (B_2) restored normal fat absorption in animals poisoned with iodoacetate (Laszt and Verzár, 1936b). Mobilization of fat and production of fatty livers in rats was inhibited by adrenalectomy and restored to normal by cortical extracts and by flavin phosphate and yeast. Althausen, Anderson and Stockholm (1939) threw an interesting light on the function of the adrenals in phosphorylation. Adrenalectomy, as always, markedly impaired the intestinal absorption of dextrose but the rate was restored to normal by salt (NaCl). Similar results were obtained in the absorption of fat by Barnes, Wick, Miller and MacKay (1939) who found fat absorption normal in adrenalectomized,

salt-fed rats. Apparently it was not the lack of cortical hormone which inhibited phosphorylation but the resulting loss of salt. Süllmann and Wilbrandt (1934) found phospholipid increased greatly in the intestinal lymph of rabbits during fat absorption so that the fatty acids in phospholipid combination constituted about $\frac{1}{6}$ of the total fatty acids. Most of the absorbed fatty acids were, however, in the form of fat so that they must have been changed to neutral fat at some stage in their absorption. The high value for the phospholipid in the intestinal lymph found by Süllmann and Wilbrandt is apparently not found in thoracic duct lymph, the phospholipid values being ordinarily very much lower. Thus, during fat absorption, the phospholipid content of the thoracic duct lymph was increased (30 per cent) although relatively little in comparison with the neutral fat increase (400 per cent) (Brockett, Spiers and Himwich, 1934). The proportionate increase of fatty acids in phospholipid to fatty acids in neutral fat was about 1 to 22 so that only about 5 per cent of the extra fatty acid was carried as phospholipid.

Using an easily identifiable fatty acid, elaidic acid, Sinclair (1935) in addition to and in support of what he had shown previously was able to show that there are in the liver probably two types of phospholipid: the structural or functional and the metabolic. The structural phospholipids contain characteristically the more highly unsaturated acids, linoleic, arachidonic, and clupanodonic and are used up only by the processes of wear and tear, while the metabolic phospholipids contain the ordinary food fatty acids and serve in the transport and combustion of the fatty acids of the food fat. This second class exhibits a rapid turnover and when the intake of fat is great may show increases in amount (Artom, 1933b; Sinclair, 1935b). It is mainly into this part of the liver phospholipids that elaidic acid and presumably other food fat enters. This part of the phospholipid is being constantly given off to the blood and carried to the rest of the body where it is presumably burned. That this part of the phospholipid is metabolic rather than structural in most tissues (e.g., muscle and brain) is shown by the slow rate at which food fatty acid enters and leaves these phospholipids as compared with the rate at which it enters and leaves the phospholipids of the liver, the intestinal mucosa and to a less extent the kidney.

These results were confirmed and extended by a series of experiments by different investigators using radioactive phosphorus, P^{32}, which entering the phospholipid during its synthesis served as a tracer in following its progress. Artom, Sarzana and Segré (1938) fed radioactive phosphorus to rats on carbohydrate and fat diets. In both cases

the amount of active phosphorus in the tissues varied with the tissue, and fat in the diet accentuated the difference. Liver and intestine had the highest values; then kidney, lungs and spleen; and finally the heart, testicles and muscles with nervous tissue lowest. In long-continued experiments, the difference faded out, indicating a general distribution with time. Ingested fat increased the synthesis of phospholipid in the liver and intestine while in other tissues the difference though present was slight, indicating that it was probably secondary, the result of absorption from the blood which transports the newly synthesized phospholipid. In the cells of the intestinal mucosa most of the newly formed phospholipid is transformed into fat again, but some of it may pass into the blood. Synthesis of radioactive phospholipid from inorganic phosphorus has been accomplished in the surviving perfused liver by Hahn and Hevesy (1938) and in intestine, liver and kidney slices by Robinson, Perlman, Ruben and Chaikoff (1938). Chaikoff and associates (Fries, Ruben, Perlman and Chaikoff, 1938) by the use of radioactive phosphorus have shown that tissues vary greatly in their phospholipid turnover. Three tissues, the intestinal tract, the liver and the kidney, were particularly active showing a maximum content of the radioactive phospholipid in from 6 to 12 hours after ingestion of the labelled phosphate. Other tissues showed a slower rise which continued for many hours, e.g., in the brain for at least 200 hours, followed by a slow decrease. At 800 hours the brain was still 70 per cent of what it was at 200 hours (Changus, Chaikoff and Ruben, 1938). This picture of phospholipid activity is very similar to that obtained by Sinclair using elaidic acid and indicates that these three tissues regularly form phospholipid from ingested fat and that this phospholipid is later carried to the other tissues, constituting an important part of the mechanism of fatty acid transport. The formation of phospholipid by liver and intestine takes place even in fasting, indicating that the stored fat when mobilized undergoes the same processes as the fat absorbed from the intestine. Judged by the evidence from fatty livers (see p. 9) an adequate exogenous supply of choline seems to be essential for continued phospholipid formation and hence for the disposal of fat and cholesterol esters.

The participation of the kidney in the rapid changes in the phospholipid during fat absorption or mobilization as shown by Sinclair and by Chaikoff and associates and confirmed in unpublished work by Haven from this laboratory is an unexpected development which lacks any adequate explanation. It is known that the kidney excretes phosphate

as part of the mechanism for the removal of excess acid and as part of its base-sparing activity, and it is possible that the phospholipid takes part in that process. Support for this conception is furnished by unpublished work of Weissberger from this laboratory. Using radioactive phosphorus along with ammonium chloride, she has been able to show an increased turnover by the kidney of phospholipid as well as inorganic phosphate. The fact noted by Griffith and Wade (1939) that choline deficiency results in hemorrhagic degeneration of the kidneys adds weight to this idea and offers an indication that phospholipids take part in all processes involving phosphoric acid.

The major part of the phospholipid turnover by the gastrointestinal tract is performed by the small intestine, the stomach and large intestine being relatively unimportant. The removal of the gastrointestinal tract and kidneys does not markedly influence the phospholipid.

TRANSPORT ACROSS THE PLACENTA AND EGG MEMBRANES. *Placenta.* Sinclair (1933) showed that the placenta of the rat is permeable to the highly unsaturated acids of cod liver oil as it is also to elaidic acid (McConnell and Sinclair, 1937); when trielaidin was fed to the mothers, the new-born young contained 16 per cent of their liver phospholipid fatty acids and 11 per cent of their whole body fatty acids as elaidic acid. After 10 days' suckling, the body fatty acids had risen to 61 per cent elaidic acid and the liver phospholipid fatty acids to 27 per cent, showing a ready passage of this fat into the milk. Bickenbach and Rupp (1931) found that the placenta of the rabbit was permeable to the unsaturated acids of linseed oil. Sinclair is careful to note that the placentas of the rat and rabbit are of the simplest type, only the endothelium of the fetal blood vessels separating the fetal from the maternal blood, a fact which made it unwise to conclude that the more complicated human placenta is permeable to lipids. However, Boyd and Wilson (1935) were able to supply some of the necessary proof. These workers found that the human placenta at full term adds considerable lipid to the umbilical blood from which it is absorbed by the fetus. The average large, well-nourished infant was found to absorb about 50 grams of lipid per day from umbilical blood, 40 grams being phospholipid and the remainder equally divided between free cholesterol and cholesterol esters. Two possible explanations were offered regarding the manner in which this transfer took place. First, the lipids may be simply passed across the placental membrane to the umbilical blood, and second, the placenta may act as a secreting gland taking up lipids from the maternal blood stream and passing them on, with or without modifi-

cation, to the fetal circulation. Later work by Boyd (1935) with rabbits supported the second explanation, i.e., that the tissue substance of the placenta was actively engaged in the *transfer of at least a part of the* lipid.

The hen's egg. The entire food of the growing chick embryo is contained in the surrounding egg and largely in the immediately adjacent yolk. The yolk consists mainly of lipids made up of about $\frac{2}{3}$ fat and cholesterol and about $\frac{1}{3}$ phospholipid. Much of this material is used up during the growth of the chick. Are these materials absorbed by simple diffusion through the fetal membranes or is there a process of digestion and absorption or secretion involving making over and modification of the yolk lipids in the transfer from food depot to living tissue? The phospholipid which appears in the embryo is apparently new phospholipid and not the phospholipid of the egg yolk. Hevesy, Levi and Rebbe (1938) introduced radioactive phosphorus as phosphate into the egg before incubation and then examined its distribution after 6, 11, 16 and 18 days. They found the phospholipid in the embryo highly active while that of the yolk was inactive, indicating that the embryo synthesized its phospholipid anew. Kugler (1936) found that lecithin and cephalin values paralleled each other both in yolk (decrease) and in embryo (increase) preserving the initial ratio of 3 lecithin to 1 cephalin, which means according to the results of Hevesy and associates that these compounds were resynthesized in the embryo in the same proportions as in the yolk. They need not be the same phospholipids, since Eaves (1910) showed that the embryo after about the tenth day can desaturate fatty acids. Hanes (1912) found that cholesterol esters began to accumulate in the liver of the developing chick about the fourteenth day of incubation, at which time a large movement of fatty material from the yolk to the embryo took place. Since there was only free cholesterol in the yolk, this must have been transported into the embryo and combined with the fatty acids at about the time that there was a large breakdown of phospholipid. Hanes' conception of the chain of events as regards the fatty acids was as follows: 2 phospholipid + cholesterol = fat + cholesterol ester.

Cholesterol ester as a fatty acid carrier. Two-thirds or more of the cholesterol of the plasma is combined with fatty acids as esters which means that, in terms of average values, about 100 mgm. per cent or $\frac{1}{4}$ of the total fatty acids or about $\frac{1}{3}$ of the value for the neutral fat of the plasma is carried as cholesterol esters. Boyd (1937) in presenting a study of a wide range of plasma lipid values showed that neutral fat,

fasting lymph, which led them to the conclusion that cholesterol aids in the absorption of fatty acids. Esterification occurred in the intestinal mucous membrane.

TRANSPORT AS NEUTRAL FAT. The presence of fat in the blood in the form of very fine particles has been known for a long time. These particles are mostly from ½ to 1 μ in diameter, are known to originate mainly from the intestine by way of the chyle, and are recognized as fat in process of transport. Most of the fat absorbed from the intestine reaches the tissues in this form and is removed and stored as fat by a process of which the details are unknown. When the removal process is interfered with, the fat particles accumulate in the blood producing the condition known as lipemia. The essential feature of this condition is the increase in the finely suspended fat which is however accompanied or followed by relatively small increases of the other blood lipids,—phospholipid, cholesterol and cholesterol esters. During fasting and especially with exercise (Gage and Fish, 1924) there is a mobilization of fat in this same finely divided form, and presumably whenever fat is mobilized from the stores it assumes this form. Barrett, Best and Ridout (1938) using heavy hydrogen, D_2, as a labelling agent were able to show that the neutral fat which accumulates in the liver originates in the fat depots: 1, in fasting; 2, after anterior pituitary extracts, and 3, after carbon tetrachloride poisoning in mice, while after a high-carbohydrate, low-protein diet or after a high-protein diet the liver fat is not the fat of the depots but is apparently that formed from carbohydrate or the protein. While the mobilized fat is known to proceed to the liver, the recent investigations of Ivy and associates (Rony, Mortimer and Ivy, 1933) indicate that it may also go to the intestine since the thoracic duct lymph in dogs fasted 2 to 14 days was found to contain 0.25 to 1.3 per cent of total fatty acids while the average content 24 hours after a fat meal was 0.2 per cent. Since there was no increase after removal of the intestine, this extra lipid must have been derived from the intestine and must have come to the intestine from the fat stores.

The purpose of mobilization of fat to the liver and to the intestine has until recently not been known, although various hypothetical explanations have been offered, such as the desaturation and phosphorylation hypothesis of Leathes. Work reviewed in this paper (see phosphorylation) shows that both liver and intestine do phosphorylate fat, i.e., change it to phospholipid, and it is therefore possible that the mobilization of neutral fat to the liver and intestine is mainly for this

purpose. During the process, there is probably a rearrangement of the fatty acids in the glyceride molecule to produce the standard tissue phospholipid pattern of about one saturated acid to three unsaturated acids per double molecule noted by Bloor (1928) and by Sinclair (1935a). Since the esterification of cholesterol with the fatty acids is known to occur during absorption from the intestine, another reason for the mobilization may be the esterification of cholesterol.

A study of the tiny fat particles (chylomicrons) in blood has been made by Ludlum, Taft and Nugent (1930) who think that the fat globules are stabilized by a protein film, since the first zone of aggregation of the particles occurs at a pH between 4.7 and 5.3, which are approximately the isoelectric points of albumin and globulin, respectively, and coalescence takes place when the acid is strong enough to precipitate the protein and destroy the film. Palmer and Wiese (1933) found that the membrane surrounding the globules of fat in milk was composed of a mixture of protein and phospholipid of which the protein was not the same as the other milk protein and the phospholipid consisted of both mono and diamino phospholipids. The relation between protein and phospholipid was not constant. The membrane was removed by churning.

Material for the formation of milk fat and probably also of egg fat is apparently transported as neutral fat, since other means of origin of the milk fat from lipids seem to be ruled out. The work of Gage and Fish (1924) with stained fat indicates that in carnivorous animals the transfer of fat from blood to milk is immediate and rapid while in the herbivorous animals the transfer is much slower. The interchange of neutral fat through the human placenta is noted by Boyd (see under placenta). The claim by Meigs and associates (1919) that blood phospholipid is a source of milk fat seems to be refuted by the work of all later investigators (Graham, Jones and Kay, 1936; McCay and Maynard, 1935). The idea that cholesterol esters of blood serve as a source of the unsaturated fatty acids of milk was advanced by Schaible (1932) who reported that the amount of unsaturated acids in cholesterol esters was greater than that in the phospholipid. The blood of lactating cows has a much higher lipid content than that of non-lactating cows or steers and the increased value is mainly in the neutral fat and phospholipid (Schaible, 1932). Maynard, McCay and Madsen (1936) found that the degree of unsaturation of the blood lipids and milk fat followed closely changes in the degree of unsaturation of the food fat. After the shift in dietary fat, there was a marked change in the iodine

HAHN, L. AND G. HEVESY. Kgl. Danske Videnskab. Selskab, Biol. Medd., **14**: no. 2, 1938.
HAVEN, F. L. AND W. F. BALE. J. Biol. Chem. **129**: 23, 1939.
HEVESY, G. Nature **136**: 754, 1935.
HEVESY, G. C., H. B. LEVI AND O. H. REBBE. Biochem. J. **32**: 2147, 1938.
JEKER, L. Pflüger's Arch. **237**: 1, 1936.
KAUFMANN, C. AND E. LEHMANN. Virchow's Arch. **261**: 623, 1926.
KITAGAWA, R. Tohoku J. Exper. Med. **24**: 329, 1934.
KLEIN, W. Ztschr. physiol. Chem. **254**: 1, 1938.
KNUDSON, A. J. Biol. Chem. **32**: 337, 1917.
KOHL, M. F. F. J. Biol. Chem. **126**: 709, 1938.
KUGLER, O. E. Am. J. Physiol. **115**: 287, 1936.
LASZT, L. AND F. VERZÁR. Biochem. Ztschr. **285**: 356, 1936a.
Biochem. Ztschr. **288**: 351, 1936b.
LOIZIDES, P. A. Biochem. J. **32**: 1345, 1938.
LORENZ, F. W., I. L. CHAIKOFF AND C. ENTENMAN. J. Biol. Chem. **123**: 577, 1938.
LORENZ, F. W., C. ENTENMAN AND I. L. CHAIKOFF. J. Biol. Chem. **122**: 619, 1938.
LUDLUM, S. D., A. E. TAFT AND R. L. NUGENT. Colloid Symposium Annual **7**: 233, 1930.
MCCAY, C. M. AND L. A. MAYNARD. J. Biol. Chem. **109**: 29, 1935.
MCCONNELL, K. P. AND R. G. SINCLAIR. J. Biol. Chem. **118**: 123, 1937.
MACKAY, E. M. AND R. H. BARNES. Proc. Soc. Exper. Biol. and Med. **38**: 410, 1938a.
Proc. Soc. Exper. Biol. and Med. **38**: 803, 1938b.
MACLACHLAN, P. L. J. Biol. Chem. **114**: 185, 1936.
MACLACHLAN, P. L. AND H. C. HODGE. J. Biol. Chem. **127**: 721, 1939.
MAN, E. B. AND J. P. PETERS. J. Clin. Investigation **12**: 1031, 1933.
MARBLE, A., M. E. FIELD, C. K. DRINKER AND R. M. SMITH. Am. J. Physiol. **109**: 467, 1934.
MAYNARD, L. A., C. M. MCCAY AND L. L. MADSEN. J. Dairy Science **19**: 49, 1936.
MEIGS, E. B., N. R. BLATHERWICK AND C. A. CAREY. J. Biol. Chem. **37**: 1, 1919.
MILLER, E. S., R. H. BARNES, J. P. KASS AND G. O. BURR. Proc. Soc. Exper. Biol. and Med. **41**: 485, 1939.
MONTGOMERY, M. L., C. ENTENMAN AND I. L. CHAIKOFF. J. Biol. Chem. **128**: 387, 1939.
MÜLLER, A. Biochem. Ztschr. **249**: 189, 1932.
OKEY, R. AND E. YOKELA. J. Nutrition **11**: 463, 1936.
ONOZAKI, T. Tohoku J. Exper. Med. **29**: 224, 1936.
PALMER, L. S. AND H. F. WIESE. J. Dairy Science **16**: 41, 1933.
PERLMAN, I. AND I. L. CHAIKOFF. J. Biol. Chem. **127**: 211, 1939a.
J. Biol. Chem. **128**: 735, 1939b.
PLATT, A. P. Biochem. J. **33**: 505, 1939.
RALLI, E. P., S. H. RUBIN AND C. H. PRESENT. Am. J. Physiol. **122**: 43, 1938.
RIEGEL, C., K. O. ELSOM AND I. S. RAVDIN. Am. J. Physiol. **112**: 669, 1935.
RIEGEL, C., I. S. RAVDIN AND H. J. ROSE. J. Biol. Chem. **120**: 523, 1937.
ROBINSON, A., I. PERLMAN, S. RUBEN AND I. L. CHAIKOFF. Nature **141**: 119, 1938.
RONY, H. R., B. MORTIMER AND A. C. IVY. J. Biol. Chem. **102**: 161, 1933.

ROSSI, G. Arch. di fisiol. **36**: 365, 1936.
SCHAIBLE, P. J. J. Biol. Chem. **95**: 79, 1932.
SCHOENHEIMER, R. AND D. RITTENBERG. J. Biol. Chem. **111**: 163, 1935.
SINCLAIR, R. G. J. Biol. Chem. **82**: 117, 1929.
Am. J. Physiol. **103**: 73, 1933.
J. Biol. Chem. **111**: 261, 1935a.
J. Biol. Chem. **111**: 515, 1935b.
Ann. Rev. Biochem. **6**: 245, 1937.
SPERRY, W. M. AND V. A. STOYANOFF. J. Biol. Chem. **117**: 525, 1937.
SÜLLMANN, H. AND F. VERZÁR. Biochem. Ztschr. **270**: 44, 1934.
SÜLLMANN, H. AND W. WILBRANDT. Biochem. Ztschr. **270**: 52, 1934.
SZÖRENYI, E. Biochem. Ztschr. **249**: 182, 1932.
TANGL, H. AND N. BEREND. Biochem. Ztschr. **220**: 234, 1930.
THAYSEN, T. E. H. Biochem. Ztschr. **62**: 115, 1914.
TUCKER, H. F. AND H. C. ECKSTEIN. J. Biol. Chem. **126**: 117, 1938.
VERZÁR, F. AND L. JEKER. Pflüger's Arch. **237**: 14, 1936.
VERZÁR, F. AND A. KÚTHY. Biochem. Ztschr. **210**: 265, 1929.
VERZÁR, F. AND L. LASZT. Biochem. Ztschr. **270**: 24, 1934a.
Biochem. Ztschr. **270**: 35, 1934b.
Biochem. Ztschr. **276**: 1, 1935.
WILLIAMS, H. H., B. N. ERICKSON, I. AVRIN, S. S. BERNSTEIN AND I. G. MACY. J. Biol. Chem. **123**: 111, 1938.